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The Development and Application of Liquid Chromatographic - Mass Spectrometric Methods for the Veterinary Drugs Carbadox and Olaquindox

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THE DEVELOPMENT AND APPLICATION OF LIQUID CHROMATOGRAPHIC – MASS SPECTROMETRIC METHODS FOR THE VETERINARY DRUGS CARBADOX AND OLAQUINDOX

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Abstract

The use of veterinary drugs and zootechnical feed additives has become widespread in modern agricultural practice. Each Member State of the European Union is required to monitor for residues of these compounds in animal tissue and animal products that are intended for human consumption. From 1st September, 1999, the use of the zootechnical feed additives, carbadox and olaquindox is not permitted within the European Union. An electrospray LC-MS-MS method for the confirmation of carbadox and olaquindox in meal is described. The method was developed for contamination level analysis of each drug in feed. An electrospray LC-MS-MS method for the carbadox marker residue in tissue, quinoxaline-2-carboxylic acid, is described. The method was validated over the range 3.0 - 300 $\mu g\ kg^{-1}$, to reflect the range of concentrations measured in NI National Surveillance Scheme samples. The Decision Limit and Detection Capability are also described. This tissue method was applied to the pharmacokinetic study of pigs exposed to housing contaminated by carbadox treated animals. Tentative criteria are suggested for a method that enables National Authorities to discriminate between deliberate use and accidental exposure to contaminated housing. A dual electrospray LC-MS-MS method for the carbadox and olaquindox tissue marker residues, quinoxaline-2-carboxylic acid and methyl-3-(quinoxaline-2)-carboxylic acid, respectively, are presented. The method was validated over the range 3.0 - 150 $\mu g\ kg^{-1}$, to reflect the range of possible concentrations to be measured in Northern Ireland National Surveillance Scheme samples. The Decision Limit and Detection Capability are also described. All the presented methods have been validated according to current European Union criteria for veterinary residue analysis (European Commission Decision 2002/657/EC). All the described methods are to be applied to the statutory analysis of samples taken in Northern Ireland.

Publications

Hutchinson, M. J., Young, P. B., and Kennedy, D. G., 2003, Development and validation of a method for confirmation of carbadox and olaquindox in porcine feedingstuffs using LC – electrospray MS-MS. *Food Additives and Contaminants*, submitted.

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Hutchinson, M. J., Young, P. B., and Kennedy, D. G., 2003, Quinoxaline-2-carboxylic acid in pigs: criteria to distinguish between illegal use of carbadox and environmental contamination. *Food Additives and Contaminants*, accepted.

Hutchinson, M. J., Young, P. B., and Kennedy, D. G., 2003, Development and validation of a dual method for the confirmation of quinoxaline-2-carboxylic acid (a metabolite of carbadox) and methyl-3-quinoxaline-2-carboxylic acid (a metabolite of olaquindox), in porcine liver using LC - electrospray MS-MS, *Journal of Chromatography B,* submitted.

Abbreviations

ADI	acceptable daily intake
amu	atomic mass units
AOZ	3-amino-2-oxazolidinone
APCI	atmospheric pressure chemical ionisation
ASMS	American Society for Mass Spectrometry
°C	degrees centigrade
°C min^{-1}	degrees centigrade per minute
C_{18}	octadecylsilyl derivatised silica
CBX	carbadox
CCα	limit of analyte quantification
CCβ	limit of analyte determination
CI	chemical ionisation
CID	collision induced dissociation
CRL	Community Reference Laboratory
CSD	Chemical Surveillance Department
CV	coefficient of variation
d_7-MQCA	deuterated methyl-3-quinoxaline-2-carboxylic acid – containing seven deuterium atoms
d_4-QCA	deuterated quinoxaline-2-carboxylic acid – containing four deuterium atoms
DAL	differential action limit
DARDNI	Department of Agriculture and Rural Development for Northern Ireland

desoxy-CBX	desoxycarbadox
desoxy-OQX	desoxyolaquindox
DHEA	dehydroepiandrosterone
DMF	N,N-dimethylformamide
DMZ	dimetridazole
DNC	4,4'-dinitrocarbanilide
EI	electron-impact ionisation
ELISA	enzyme linked immunosorbent assay
et al.	and others
EU	European Union
eV	electron volts
FAO	Food and Agriculture Organisation of the United Nations
Fig.	Figure
g	gram(s)
g	acceleration due to gravity
$g\ l^{-1}$	grams per litre
h	hour(s)
HCl	hydrochloric acid
GB	Great Britain
GC-MS	gas chromatography – mass spectrometry
GSH	reduced glutathione
GSSG	glutathione
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HR-MS	high resolution – mass spectrometry

IP	identification point(s)
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	kilogram(s)
l	litre(s)
$l\ h^{-1}$	litres per hour
LC-MS	liquid chromatography – mass spectrometry
LoQ	limit of quantification
LR-MS	low resolution – mass spectrometry
Ltd.	Limited
M	moles per litre
mbar	millibars of pressure
mg	milligram(s)
$mg\ kg^{-1}$	milligrams per kilogram
$mg\ kg^{-1}\ day^{-1}$	milligrams per kilogram per day
$mg\ ml^{-1}$	milligrams per millilitre
$mg\ l^{-1}$	milligrams per litre
MIC	minimum inhibitory concentration
min	minute(s)
ml	millilitre(s)
$ml\ min^{-1}$	millilitres per minute
mm	millimetre(s)
mM	millimoles per litre
monoxy-CBX	monoxycarbadox
monoxy-OLQ	monoxyolaquindox
MQCA	methyl-3-(quinoxaline-2)- carboxylic acid

M_r	molecular mass
MRL	maximum residue limit
MRM	multiple reaction monitoring
MRPL	minimum required performance limit
ms	millisecond(s)
MS-MS	tandem mass spectrometry – mass spectrometry
m.w	molecular weight
m/z	mass per unit charge
n	number
ηg	nanogram(s)
ηg g^{-1}	nanograms per gram
ηg ml^{-1}	nanograms per millilitre
ηg l^{-1}	nanograms per litre
nm	nanometre(s)
NI	Northern Ireland
NPAOZ	3-([2-nitrophenyl)-methylene]-amino)-2-oxazolidinone
NOEL	No Observable Effect Level
NRL	National Reference Laboratory
NSAID	non-steriodal ant-inflammatory drug(s)
NSS	National Surveillance scheme
OLQ	olaquindox
pH	scale of acidity
pK_a	scale of charge
QCA	quinoxaline-2-carboxylic acid
QDN	quindoxin

-R	attached side-chain
r	correlation coefficient
RAS	renin-angiotensin system
RIA	radioimmunoassay
RIVM	National institute of public health and environment in The Netherlands
rpm	revolutions per minute
RSD	relative standard deviation also known as the coefficient of variation
s	standard deviation
SCAN	Scientific Committee on Animal Nutrition for the European Union
SCX	benzenesulfonic acid
sec	second(s)
SEM	standard error of the mean
SIM	selected ion monitoring
SPE	solid phase extraction
spp.	species
S_r	relative standard deviation
t	time
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TR-FIA	time-resolved fluoroimmunoassay
TRIS	tris(hydroxymethyl)methylamine
UK	United Kingdom

USA	United States of America
UV	ultra-violet
µA	micro amp(s)
µg	microgram(s)
µg g^{-1}	micrograms per gram
µg kg^{-1}	micrograms per kilogram
µg l^{-1}	micrograms per litre
µl	microlitre(s)
V	volt(s)
Vis	visible
VSD	Veterinary Sciences Division
v/v	volume per unit volume
w/v	weight per unit volume
w/w	weight per unit weight
WHO	World Health Organisation
%	Percentage

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Chapter 1

General Introduction

1.1. Overview

Since the 1960s there have been dramatic increases in the range and diversity of foods we eat through global marketing and expanded international trade. This has led to massive changes in the methods of food production - no longer is it possible to rely on small producers to meet the demands of the population and still have a viable export market. Producers now strive to use high intensity, highly efficient, and low cost methods of production to provide for the current demand. It was the unexpected discovery in the 1950s that antibiotic drugs increased growth rates and improved the efficiency of food utilisation in pigs and chickens that has led to their use as growth promoters ever since. This has greatly aided the evolution of such intensive production methods for livestock. The discovery of the growth promoting action was through their use at sub-therapeutic levels, mainly as additives to feed. Such use was as a prophylactic for the prevention of common diseases (to which intensively reared animals are susceptible) and the rapid spread of infectious disease. A wide range of drugs is available to veterinarians and producers for use in modern agriculture. They are used for the management of specific disease states, treatment of parasitic infection, prevention of inflammatory reactions, and many others. However, since the 1950s they have found widespread use as growth promoters and prophylactics.

Since the early 1980s, there has been greatly increased public awareness of health issues in general, as well as a growing interest in animal health welfare and ethical issues. Concern has been expressed regarding the impact on public health from the use of drugs in animals for human consumption, and the intensive methods used in modern food production. These concerns arise from consumer confidence in the wholesomeness of food having been destabilised through several large public health scandals. One good example

of this has been the Salmonella outbreaks in eggs in the early 1990s, which decreased egg consumption over the next several years. The public is now more aware than ever that what they eat is not necessarily either wholesome or good for their health. Reports as early as 1969 concluded that the administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, posed hazards to both human and animal health (Swann, 1969). Many reports have also been published linking resistant strains of bacteria and allergic reactions to veterinary drug residues, as well as queries over their long-term physiological effects in humans, through the consumption of such residues. This has led to much criticism and debate in the media over modern farming methods and the use of growth promoting products in agriculture.

Contrary to popular opinion, agricultural use of antibiotics is extensively regulated. Legislation has been in place since the mid. 1970s in the European Union (EU), governing the use of veterinary medicines and zootechnical feed additives. Legislation, to which all Member States within the EU must adhere, is in place concerning the monitoring of residues of such products, to guarantee the safety and quality of animals and animal products for human consumption. Each country within the EU is responsible for its own residue testing, producer sampling, and National Surveillance Schemes, all of which must comply with current EU regulations. Such systems involve the expenditure of considerable resources in terms of finance and labour per annum. The British residue surveillance scheme cost the government £3.6 million in 2001 for statutory testing alone (Veterinary Residues Committee, 2001). Active research is also carried out to improve and develop new analytical methods for the detection of drug residues, plus investigations into the pharmacokinetics of these drugs, which can help analysis and enforcement of any current bans on use.

1.2. Veterinary drugs

In agriculture, either a bacterial or a parasitic agent is the most common cause of disease. Anti-infective drugs are largely classified into groups according to their function – as either an anti-infectious or an anti-parasitic agent, rather than by their chemical structure as in human pharmacology. Within these groups, both specific function and chemical structure may further sub-classify the drugs. Anti-infective agents are classed into antibacterial, antifungal, antiviral, and antiprotozoal agents. From the point of view of drug residues and monitoring, antibacterials and antiprotozoal drugs are the most important, due to the sheer volume of their use.

1.2.1. Antibacterial drugs

Antibacterial drugs, which include natural, semi-synthetic and synthetic compounds, consist of bactericidal agents that kill bacteria, and bacteriostatic agents that inhibit cellular neogenesis allowing the immune response to kill the infecting bacteria. They are classified into 10 major groups: β-lactams, tetracyclines, aminoglycosides, macrolides and lincosamides, amphenicols, sulphonamides and potentiated suphonamides, nitrofurans, nitroimidazoles, quinolones, and 'other' drugs. Larger groups such as the β-lactams have been further sub-divided on their function against disease states. Commonly used antibacterial drugs include penicillin, tetracycline, neomycin, and trimethoprim all of which have use in both veterinary and human medicine.

1.2.2. Antiprotozoal drugs

Antiprotozoal agents are classified into several major groups - based on the causative protozoa. Anticoccidials (e.g. monensin, narasin and lasalocid) are active against *Eimeria*

or *Isospora* spp. Drugs for histomoniosis (e.g. dimetridazole and nifursol) which are active against *Histomonas meleagridis*. Drugs for trichomoniosis (e.g. ronidazole and carnidazole) are active against trichomonads. Drugs for babesiosis (e.g. Imidocarb dipropionate and diminazine) are active against *Babesia* spp. Drugs for giardiosis (e.g. fendendazole) are active against *Giardia* spp. Drugs for hexamitiosis (e.g. dimetridazole) which are active against *Hexamita meleagridis.* Drugs for leishmaniosis (e.g. pentamidine and allopurinol) are active against *Leishmania* spp. Drugs for theileriosis (e.g. buparvaquone and chlortetracycline) are active against *Theileria* spp. and drugs for typanosomosis (e.g. diminazine).

There exists some crossover between drug groups and classes and they are by no means exclusive. For example nitroimidazoles can be used both as an antibacterial agent in the treatment of infections caused by anaerobic bacteria as well as in the treatment of giardiasis. 4 -Hydroxyquinolones such as oxolinic, pipemidic and nalidixic acid can be used against gram-negative bacterial infections as well as in specific protozoal infection.

1.2.3. Antiparasitic drugs

Antiparasitic agents are a group of drugs used in the treatment and control of parasitic infections caused by protozoa, helminths, and arthropods. These are used as either endoparasiticides, for the treatment of internal parasitic infections or ectoparasiticides, for the treatment of external parasitic infections. They are classified according to the causative helminth and are split into 3 groups: drugs for nematodes (roundworm), drugs for cestodes (tapeworm), and drugs for trematodes (flukes). These agents vary substantially in chemical structure and mode of action. They include avermectins, milbemycins, benzimidazoles, imidazothiazoles, and organophosphates. These same agents and others,

such as benzoyl urea derivatives and carbamates, are also used as ectoparasiticides via suspension in topical and dipping solutions or as powders.

1.2.4. Administration of veterinary drugs

There is great variation in the routes of administration of these veterinary medicines – muscular or subcutaneous injection, implanted pellet, oral drenching, bolus, or pour on preparations. The most commonly used route of administration is oral, achieved by incorporating the medication in milk replacer, water, or feed. Low medication levels of antibiotics have been used in livestock production to improve feeding efficiency and increase weight gain. Estimates of the beneficial effects vary, but such sub-therapeutic use in young swine has been reported to increase the average daily feed efficiency from 6 to 8% and the average daily weight gains from 10 to 23% (Prescott, 1997). Sub-therapeutic levels are also used as preventative measures against commonly occurring diseases. For example, tetracyclines have been used in the poultry industry to prevent bacterial infections in flocks. Higher medication levels can be used above a bacterium's Minimal Inhibitory Concentration (MIC) as treatment for a disease state.

The worldwide use of veterinary drugs as feed additives is extensive, with the market for antibacterial and anticoccidial worth several billion dollars a year. In addition to their legal use as licensed medicines, unscrupulous producers have been found to use some compounds illegally as growth promoters. For example, the β-agonist clenbuterol has growth promoting effects when used at five times the recommended concentration for the treatment of respiratory disease. A major cause of public concern in the past few years is the presence of veterinary drugs, their residues, and other chemical contaminants in animals for human consumption at the time of slaughter, as well as in milk and eggs.

Consequently, the potential exists that this may give rise to human health problems, possible side effects or illnesses as the long-term toxicological effects are not known, in addition to increases in the incidence of antibiotic resistant bacteria. Incidences of food related scares are usually well reported in the media as they are seen as human-interest stories.

1.3. Veterinary drug residues

The EU has defined veterinary drug residues as:

"- All pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in food stuffs obtained from animals to which the veterinary medicinal product has been administered." (European Commission, 1990a).

EU Council Regulation No 2377/90 (see Section 1.7.2.) lists the maximum legally permitted concentration of residues resulting from the use of a veterinary medicinal product that is acceptable by the EU in animal tissue. This is known as the Maximum Residue Limit (MRL) and has been established due to concerns over both short and long-term effects on human health. MRLs are only set for compounds that are legally licensed for use within the community. For previously unlicensed compounds i.e. compounds that have had their licence withdrawn, no MRL is set, as any traces of such residues are not permitted in animal tissue.

Residues resulting from the use of a veterinary medicinal product are normally formed when the compound is metabolised into a water-soluble product to allow excretion via

urine or bile / faecal matter. Drug metabolism is normally divided into two parts: Phase I and Phase II.

Phase I or functionalisation reactions

Phase I is commonly thought of as a preparation step for Phase II. These include oxidation, reduction, hydrolysis and hydration reactions. The majority of these are carried out by the microsomal mixed-function oxidase systems that are cytochrome P450 dependent in the endoplasmic reticulum of cells most notably in the liver, kidney, lung and intestine. The final product usually contains a chemically reactive functional group that allows reaction with phase II enzymes. For the clearance of some drugs phase I metabolism is sufficient to allow excretion.

Phase II or conjugation reactions

Phase II usually involves formation of a water-soluble product that can be secreted in the bile or urine. This is the true 'detoxification' step. This involves conjugation with sugars, amino acids, fatty acids and cholesteryl esters, as well as sulfation, methylation, acetylation, and condensation reactions.

Some drugs do not require Phase I or Phase II reactions; chemical catalysis can occur by endogenous metabolism in the target tissue or organ that converts the parent compound to a water-soluble product. Clearance of the product is usually via kidney or the liver; the specific route largely influenced by molecular weight (m.w.). Drugs with m.w. less than 300 are largely secreted in the urine, those larger than m.w. 300 are largely secreted in the bile. As a result of these reactions, metabolites may have different chemical and biological properties than that of the parent drug. Different species may also generate more of one

metabolite through differing enzymic concentrations or iso-enzymatic forms. These factors can affect the half-life / clearance rate of a metabolite. Consequently, residues of compounds can exist in one tissue type for several days and in another for several weeks, even after the parent compound has been excreted from the system. A good example of this is the antimicrobial furazolidone, which has been used in poultry production as a growth promoter and prophylactic feed additive. Furazolidone has a very short biological half-life *in vitro* and *in vivo*, being rapidly converted within 4-6 hours to metabolites containing a 3-amino-2-oxalidinone (AOZ) side-chain that are protein bound. These AOZ residues have been detected in porcine tissues 6-7 weeks after cessation of treatment (McCracken *et al.*, 1997).

1.4. Causes of veterinary drug residues

Providing that a licensed drug is used as per product licence and the correct withdrawal times are obeyed before slaughter, violative levels of residues should not occur in animals or animal products for human consumption. However, such concentrations of residues can occur before slaughter due to a number of reasons:

1.4.1. Failure to obey withdrawal time

EU legislation lists MRLs for each licensed veterinary drug (European Commission, 1999). The withdrawal period is the interval that must elapse (after the cessation of treatment) before an animal may be slaughtered and any derived products used for human consumption. This period is designed so that any residues resulting from administration of medication will be below the MRL, at the time of slaughter. The *Animals and Animal Products (Examination for Residues and Maximum Residues Limits) Regulations* 1997 implement these regulations in the United Kingdom (UK). These state that an animal may

not be supplied or slaughtered if the withdrawal period for a legally administered veterinary product has not been obeyed. Withdrawal times are stated individually by species, as differing physiology can increase or decrease the rate at which residues are cleared from tissue. Withdrawal times can also vary depending on the individual formulation i.e. two products containing the same concentration of an active principle can have differing withdrawal periods.

To comply with the withdrawal time, unmedicated rations must be supplied to the animals throughout this period. A producer may consider this a needless expense, or this period as excessive or inconvenient as it delays the sale of livestock. This can lead to no and/or a reduced withdrawal time being followed, resulting in residue concentrations above the maximum permissible levels at slaughter.

1.4.2. Cross contamination of feed

After the preparation of medicated feed in feed mills, subsequent batches of feed may contain sub-therapeutic levels of the drug used. This may still occur after flushing of the production line, due to the electrostatic properties of the drug formulation. One study with the coccidiostat, monensin, widely used in the broiler chicken industry, reported levels greater than 5 % of the therapeutic dose in 9 of the 40 withdrawal meals tested from one feed mill. These sub-therapeutic levels may be enough to cause 'violative' residues in the tissues, eggs or milk of an animal if fed such contaminated rations during a withdrawal period (Kennedy *et al.*, 1998c). Another study tested 397 feedingstuffs and 11 premixes of which 35.2 % were found to contained undeclared antimicrobials. The most frequently identified were chlortetracycline, sulphonamides, penicillin, and ionophores. All the contaminating concentrations of sulphadimidine detected would have been sufficient to

cause tissue residue levels above the MRL, if fed to animals as withdrawal rations prior to slaughter (Lynas *et al.*, 1998). Manufacturers have addressed these problems by producing granular medicated preparations that have greatly reduced electrostatic properties. This reduces the degree of carryover, but does not eliminate the problem. For example the introduction of a granular form of medicated premix for the coccidiostat lasalocid reduced the incidences of lasalocid residues in eggs in Northern Ireland, but did not eliminate the problem entirely (Kennedy *et al.*, 1998a).

1.4.3. On-farm contamination

On-farm contamination may occur by the same means as in industry (see Section 1.4.2.), if the medicated feed is prepared on-site and inadequate purging of the system takes place. Short-term exposure before slaughter of previously unmedicated animals to housing that has contained treated animals has been shown to result in residues in tissue. Residues of furazolidone, a nitrofuran antibacterial effective against *Escherichia coli* and *Salmonella* spp. infections in pigs, have been found in unmedicated pigs following exposure to a cleaned housing that had previously contained pigs undergoing furazolidone medication (McCracken *et al.*, 1997). Similar findings have been reported for residues of sulphadimidine, used in intensive pig production for the prevention of many respiratory and gastrointestinal diseases (Elliott *et al.*, 1994b).

1.4.4. Illegal or improper use of drugs

Improper use of a drug above the recommended therapeutic concentration, as listed in the current UK Veterinary formulary (Anonymous, 2001), can lead to residues existing even after the observation of the correct withdrawal time for the drug. This may also be applied

to the use of a veterinary medicine authorised for use in another animal species or for another condition in the same species, known as 'Off-label use'. Illegal use of drugs, such as hormones for the purpose of increasing live-weight gain, may give rise to residues at slaughter even after a significant withdrawal period (Meyer and Rinke, 1991).

Annex I and III of EU Council Regulation No 2377/90 lists the MRL concentrations for all permissible veterinary compounds. If a producer can give no valid reason to justify a result exceeding the MRL set for a compound, further sampling maybe carried out. This can lead to condemnation of animals and ultimately prosecution in a court of law. The same criterion applies following a positive result for a banned drug (compounds which are listed in Annex IV of the same regulation), and other unlicensed substances (e.g. zootechnical compounds that have had their licences withdrawn).

1.5. Risks of veterinary drugs and their residues to public health

In Great Britain (GB) in 2001, ~39,000 samples of meat were collected and tested under the National Surveillance Scheme (Veterinary Residues Committee, 2001). Of the ~44,000 analyses performed, only 155 revealed samples which contained veterinary drug residues above the MRL or Differential Action Level (DAL). NI operates a separate sampling system linked to GB. In 2000, 3,334 samples of meat were taken for analysis under the NI national plan for veterinary drug residue surveillance. Of these 3320 (99.6%) were found to be free from violative residues. 14 (0.4%) of these samples contained residues in excess of the MRL or DAL (Veterinary Medicines Directorate, 2000). Though the problem of residues is statistically small, it still poses a significant threat to public health. With the wide range of veterinary drugs and products available to producer, the potential exists for chemical residues to be present in animal products destined for human

consumption, leading to possible short and long term pharmacological side effects. Though the number of assessed positive samples is low, each positive is a representative sample taken randomly from a large group of animals at slaughter, the derived products of which can be consumed by a significant number of the population. These veterinary drugs and their residues have been shown to give rise to several potential threats to the consumer and producer.

1.5.1. Acute pharmacological effects

Clenbuterol, popularly known as 'Angel Dust', is the most commonly abused of the β-agonists. Its action as a growth promoter requires concentrations in excess of five times the recommended therapeutic dose for respiratory disease, for extended periods (>30 days). This alters the fat to muscle deposition ratio and increases the live weight (Ricks *et al.*, 1984), consequently increasing the price per head of livestock for the producer at sale. Such elevated medication levels can result in accumulation of significant concentrations of the drug in edible tissues (Meyer and Rinke, 1991). High concentrations of such drug residues in animals and animal products have resulted in the poisoning of consumers, giving rise to symptoms such as muscle tremors, palpitations, tachycardia and myalgia that have lasted up to forty hours (Martinez-Navarro, 1990; Pulse *et al.*, 1991). Similar risks may be associated with the consumption of injection sites and implanted pellets, which contain high local concentrations of the drug in the tissue.

1.5.2. Allergies

Reactions to drugs and their residues include photosensitivity, photoirritation, inflammatory reactions, and in severe cases - anaphylactic shock. Such possible allergic

reactions must be differentiated from reactions to natural food constituents, such as to the oils present in nuts. β-Lactams, though relatively non-toxic, may be responsible for most of the reported human allergic reactions to antibiotics. As early as 1959, allergic reactions to penicillin and its derivatives were recognised. Penicillin was used extensively at that time to treat mastitis in dairy animals. As a result of non-compliance with recommended withdrawal times, penicillin had become a common adulterant in marketed milk. Allergic reactions, such as skin rashes, to contaminated milk were recognised at this time by public health authorities (Zimmerman, 1959). Skin allergies have been shown to occur through direct contact with pure drug in feed mills or through 'on-farm' handling of medicated feed without protective equipment. The use or exposure of humans to several drugs, particularly diuretics, neuroleptics and antibiotics, has been well documented to induce photoirritation and/or photoallergic reactions (Boulitrop-Morvan *et al.*, 1993; Mizuma *et al.*, 1999; Tokura, 1998). Of the antibiotics, quinolones and fluoroquinolones have the greatest potential for inducing photoirritancy and have been documented in a variety of experimental studies (Christ and Esch, 1991).

1.5.3. Carcinogenic and mutagenic effects

Veterinary drug residues occur in tissue at such low levels that they rarely pose chronic or long-term hazards to human health. Although the parent compounds of certain drugs may be relatively innocuous and chemically inert (though clinically active), their metabolites have been shown to exhibit carcinogenic and mutagenic effects in laboratory animals. Aromatic hydrocarbons as well as many cyclic compounds can be metabolised by cytochrome P450 and epoxide hydrolyase to form electrophilic diol-epoxide metabolites that are capable of covalent bonding with nucleic acids i.e. chemical carcinogenesis. For example, nitroimidazoles such as metronidazole, dimetridazole and ronidazole, have been

shown to be carcinogenic in bacteria due to suspected reductive 5-nitro metabolites (Voogh *et al.*, 1974). Dimetridazole, ronidazole and ipronidazole medication has also been linked to increased incidence of mutation and tumour formation in rats (WHO/FAO, 1989). The hormone oestradiol-17-β is prohibited from use in the EU, as a substantial body of evidence exists suggesting that it is a carcinogen and that it possesses tumour-promoting effects (European Commission, 1996b). The hormones testosterone, progesterone, trenbolone acetate, zeranol and melengestrol acetate are also prohibited on suspicion of having similar effects, though they are still legally permitted in the USA. The EU recently upheld the prohibition of such substances, as they have not been able to give a quantitative estimate of the risk to consumers. The EU considers the possible long-term exposure to even low levels of veterinary residues with possible carcinogenic, mutagenic or genotoxic effects, to be an unacceptable potential risk to the consumer.

1.5.4. Antibiotic resistance

A major potential risk exists with the widespread use of antibiotics in livestock. There is the possibility of drug resistant pathogens occurring through natural selection, and subsequently that these animal pathogens may be transmitted to man. Zoonotic infections do exist, such as *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia coli* 0157:H7 ('the hamburger pathogen'). Such 'super bugs' have led to public health scares and the closure of hospital wards. Of other concern is the possible emergence of antibiotic resistant non-pathogens that can colonise humans or livestock gut flora for extended periods with no adverse effects. This gives rise to the potential transfer of resistance to bacteria capable of causing pathogenesis through the well-documented swapping of genetic elements such as plasmids. If such resistant strains were to occur in livestock, the possibility exists that resistance could be passed to human pathogens. There also is a risk

that administration of an antimicrobial used only in veterinary medicine could lead to the selection of strains resistant to antibiotics in human medicine. For example, in April 1997 the EU suspended the use avoparcin, a glycopeptide antibiotic, never used in human medicine, following the publication of results showing that its use was closely associated with the occurrence of vancomycin-resistant *Enterococcus faecium* in food animals (Møller Aarestrup *et al.*, 1996). Such was the concern over the possible transfer of resistance to human pathogens, that a Community ban was initiated, although the EU Scientific Committee for Animal Nutrition (SCAN) had already concluded in 1996 that there was no direct evidence of any link. Though the latent risk for antibiotic resistant pathogens exists as a result of the use of veterinary drugs in livestock, they cannot wholly be blamed for occurrence of resistant strains that are of current medical concern. The high, routine use of antibiotics in human medicine must take the largest portion of the blame, through the 'empirical' prescribing within community practice and prescribing 'blind' on presumption via the description of symptoms.

1.5.5. Environmental Effects

A significant proportion of veterinary drugs that are administered to animals is ultimately spread in the terrestrial and aquatic environments (Hälling-Sørensen *et al.*, 1998). The exact concentration of chemical residues that pass through an animal is dependent on species and the route of administration of the drug. For example a drug given orally has a higher chance of being present in faecal matter due to incomplete absorption in the gastrointestinal tract e.g. ampillicin. Intravenous injection is much less likely to do so as it bypasses the absorption in the gut wall, passing directly into the systemic circulation. Such compounds may persist in the environment for days or months depending on the compound in question and the temperature (Gavalchin and Katz, 1994). Detrimental

effects can be observed on non-target organisms in the soil that are required for natural environmental processes such as recycling of animal waste, nitrogen fixation, etc. Persistent residues can be transferred into the aquatic environment through surface run-off or by leeching of the soil profile, as is the case for several other chemicals such as pesticides. High concentrations in an aquatic system may ultimately lead to significant effects on algal and cyanobacterial populations (Hälling-Sørensen, 2000), fresh water crustaceans (Wollenberger *et al.*, 2000), and other fresh-water organisms (Canton and Van Esch, 1978). Similar antibiotic persistence has been shown to occur in marine sediments from the use of antibiotics in aquaculture (Hektoen *et al.*, 1995). In both the aquatic and terrestrial environments, there is again the potential for the emergence of resistant species through natural selection as previously mentioned (see Section 1.5.4.).

1.6. Establishment of maximum residue limits

The value for the MRL in food within the EU for legally licensed compounds is determined in a number of steps. The sponsor of the drug is required to carry out a wide range of toxicity, metabolic and depletion studies as part of the approval process for use. From these, the tissue from which the drug and its residues deplete the most slowly can be determined. The longest remaining metabolite in this tissue usually being named as the marker residue for the use of the drug. However, if studies show that a particular metabolite of a drug is toxic, an MRL may be established for this instead. Firstly, the Acceptable Daily Intake (ADI) in mg kg^{-1} day^{-1} for the residue must be determined. This value is the concentration of drug that can be consumed per day, during an entire lifetime with no appreciable health risk, based on all the known facts at the time. This No Observed Effect Level (NOEL) is calculated on the basis of systemic, reproductive, and developmental toxicology studies *in vitro*, and genotoxicity/mutagenicity *in vivo*. The

value is taken for most sensitive effect or for the species most likely to show the same response as humans. This is then divided by a safety factor, usually 100, to allow for variability in extrapolating from animals to humans to give the ADI. This assumes that there is a 10-fold range in sensitivity and that humans are 10 times more sensitive than animals. The MRL is calculated by dividing the ADI with a theoretical amount of meat or animal products consumed daily. These have been established by the World Health Organisation (WHO) for various tissues (WHO/FAO, 1989):

1) 50 grams of kidney
2) 100 grams of liver
3) 300 grams of muscle
4) 100 grams of eggs
5) 1.5 litres of milk

If a drug is to have a licence granted for more than one species, withdrawal times must be established for the depletion of tissue concentrations below the MRL in each. Different MRLs are calculated for various food products, like milk, to ensure that children do not consume residues at a concentration exceeding the ADI for the compound in question based on reduced bodyweight. An extra 10-fold factor is usually included in the extrapolated safety factor.

1.7. European Union legislation concerning veterinary drugs

Veterinary medicines are legally classified into general sales list medicines, pharmacy medicines, pharmacy and merchant list medicines, prescription only medicines, and controlled drugs. The use, administration, and licensing of such medicines are controlled

under national and EU regulations. Zootechnical feed additives such as production enhancers or coccidiostats are independently controlled and authorised under EU legislation, as indicated in the relevant Annex of the current amended version of EU Council Directive 70/524/EEC (European Commission, 1970). EU legislation lays down requirements and technical details for the detection and monitoring of the residues of these compounds. Part of the terms of admission, as an EU Member State is to adhere to these directives. EC Council Regulation No 2377/90 governs the legal use of veterinary drugs and the residue levels of each that are permitted. The monitoring of these residues and the steps that must be taken to guarantee and ensure the quality of import and export products to and from the EU are governed by EU Council Directive 96/23/EC.

1.7.1. European Union Council Regulation No 2377/90

This regulation establishes the MRLs in animals and animal products (European Commission, 1990a), for the drugs to be monitored listed in EU Council Directive 96/23/EC (see Section 1.7.1.). These have been split into the Annexes listed below. Each monitored substance is listed in one of the following Annexes to the regulations:

Annex I – Pharmacologically active substances for which final MRLs have been set e.g. oxytetracycline, ivermectin, and dexamethasone.

Annex II – Substances, which do not need an MRL e.g. caffeine, ethanol, and hydrocortisone.

Annex III – Pharmacologically active substances for which provisional MRLs have been fixed e.g. erythromycin, streptomycin, and clenbuterol.

Annex IV – Pharmacologically active substances for which no maximum levels can be fixed e.g. metronidazole, chloramphenicol, and chlorpromazine.

As from 1st Jan 2000, substances not listed in Annex I, II, and III cannot be administered to food producing animals or used in veterinary medicinal products. Substances listed in Annex IV are not considered safe at any level and cannot be used in livestock production. Any zootechnical feed additives that have had their licences withdrawn will not come under Annex IV and each comes under separate EU legislation.

1.7.2. European Union Council Directive 96/23/EC

This directive (European Commission, 1996c), which superseded EU Council Directive 86/469/EEC, lays down measures to be taken by Member States to monitor for substances with applications of use in the agricultural sector. Annex I lists the groups of substances that are required to be monitored. This Annex has been split into two groups of substances:

Group A - which are substances having an anabolic effect and unauthorised substances. This covers stilbenes and derivatives, antithyroid agents, steriods, resorcyclic acid lactones, β-agonists, and compounds included in Annex IV to EU Council Regulation No 2377/90.

Group B - which are veterinary drugs, including unlicensed substances used for veterinary purposes, and contaminants. This covers antibacterial substances, veterinary drugs such as anthelmintics, anticoccidials, carbamates and pyrethroids, sedatives, non-steroidal anti-inflammatory drugs (NSAIDs), other pharmacologically active substances. Also other substances and environment contaminants, such as PCBs, organophosphorous compounds, chemical elements, myotoxins, and dyes.

Annex II of same directive, lists the residue or substance groups to be detected by type of animal, feed, or product. Annexes III and IV, lay down the rules for the surveillance of such residues by animal type/product, and include sampling strategy, rules, and levels of sampling required. The directive also lists administrative measures required of every member state:

1) A public body must be assigned to be responsible for co-ordinating, monitoring, and inspections.
2) This public body must draw up a national plan for the surveillance of residues in compliance with Annex III and IV of the Directive.
3) The results of such a plan must be reported annually to the EC.
4) Each member state must have at least one National Reference Laboratory (NRL), to implement quality assurance schemes.

The Annex V of the directive also designates four Community Reference Laboratories (CRLs), each of which is responsible for a specific group of residues. The CRLs are there to help NRLs implement their quality assurance schemes, promote and co-ordinate research and development, provide technical advice and analytical methods, and to train analysts from NRLs in both current and new analytical methods.

1.8. Application of Legislation in Northern Ireland

EU Council Directive 90/167/EEC controls manufacture, distribution, and usage of medicated feeding stuff (European Commission, 1990b). This has been subsequently implemented in NI by *Feedingstuffs Regulations (Northern Ireland)* 1995 and

implemented in UK by *Medicated Feedingstuffs Regulations* 1998. The monitoring of veterinary drugs and their residues in both live animals and animal products is required under EU Council Directive 96/22/EC (European Commission, 1996b) and 96/23/EC (European Commission, 1996c). Control in NI comes under the *Animals and Animal Products (Examination for Residues and Maximum Residue Limits) Regulations (Northern Ireland)* 1998, and this act also implements EU Council Regulation No 2377/90. NI traditionally exports the majority (~70 - 80%) of its meat and meat products. The Meat Hygiene Service inspects all animals submitted for slaughter and a national sampling scheme exists to monitor for the presence of drug residues in meat. This surveillance scheme is linked to National Surveillance Scheme in GB. Additionally, two other intensive surveillance schemes are also operated within NI; the meat inspection scheme and the pig testing scheme, to enhance food safety assurance for the export-led industry in NI.

1.8.1. National surveillance scheme for Northern Ireland

Under the UK National Surveillance Scheme a specific percentage of the total samples are allocated to NI each year, in proportion to the number of the various species slaughtered the previous year. In the year 2000, 3,334 samples were taken for analysis under the National Surveillance Scheme for NI. Sampling of 1,103 cattle, 604 pigs, 259 sheep, and 638 poultry was carried out. On-farm sampling of feeding stuffs was carried out on 146 cattle, 14 pig, and 65 poultry feeds, also taken were 146 milk samples and 153 egg samples (Veterinary Medicines Directorate, 2000).

1.8.2. Meat Inspection Scheme

Under the Meat Inspection Scheme, the on-site government vet can detain animals if suspicions of drug treatment are raised, and samples submitted for analysis. Signs of drug treatment include adrenal lesions, injection / implant site marks and over-enlarged thyroid glands. Any screened samples, which give a positive result for banned compounds or a result above the permitted MRL for licensed medicines is subject to confirmatory analysis. If the result is upheld after confirmatory analysis, any carcasses linked to the sample are condemned and further on-farm sampling of the producer is carried out. Such extended sampling can ultimately lead to prosecution.

1.8.3. Pig Testing Scheme

This is a targeted sampling scheme for antimicrobial residues in pigs at slaughter. Samples are taken randomly at slaughter and submitted for screening for a wide range of drug residues. If a screened sample is positive or above the MRL, the sample is sent for confirmatory analysis. Confirmed positives lead to the detaining of up to five animals when presented for slaughter by the offending producer. Any positive result leads to condemnation of the carcasses and further sampling of any future animals submitted by that producer. In the year 2000 in NI, almost 5,000 samples were taken under the pig testing scheme, equivalent to 1 in every 250 pigs slaughtered (Veterinary Medicines Directorate, 2000).

1.9. Chemical surveillance methods

To facilitate the testing of large numbers of samples generated by National Surveillance Schemes and other monitoring programmes, a two-tier system of chemical surveillance is generally employed. Samples are first screened in large batches using rapid, high

throughput procedures with a simple negative or positive for the presence of the drug. In the case of positives, such samples are subject to confirmatory methods for unequivocal identification and quantification of the analyte. Such methods are slower and involve extended clean-up procedures, therefore requiring a longer turn around time.

1.9.1. Screening methods

Due to the large number of samples and to meet the required turn around times (~1 day) for the reporting of results, these procedures should be simple, rapid, and inexpensive. Sensitivity is required so the presence of an analyte can be detected at MRL levels or below for legal substances or a simple positive or negative for trace levels and above of a banned substance. The procedures should also have an acceptable probability of false negative results ($\leq$ 5%). Several types exist; the charm receptor assay, based on the binding between functional groups on a drug and microbial cell receptor sites, is now widely used for the detection of a particular class of antibiotics in milk. Enzyme Linked ImmunoSorbent Assays (ELISA) and RadioImmunoAssays (RIA) are also frequently employed (Elliott *et al.*, 1994a). Their usefulness is limited by the fact that they require unwanted extraction and clean-up steps for tissue samples, and each is applicable only to one class of compounds. Time – Resolved Fluorescence ImmunoAssays (TR-FIA) that are based on the unique fluorescent properties of lanthanide chelates are well established in human medicine as an *in vitro* screening technique. This technique has now been adapted for veterinary *in vitro* diagnostic screening in the analysis of steroid hormones (Elliott *et al.*, 1995) and monensin (Crooks *et al.*, 1998b) in plasma. Most recently it has been adapted to the analysis of zeranol in bovine urine (Tuomola *et al.*, 2002). High Performance Liquid Chromatography (HPLC), and Thin Layer Chromatography (TLC) are also routinely used in screening. Though not often employed, Gas Chromatography –

Mass Spectrometry (GC-MS) has been used in the screening for anabolic agents and β-agonists (Blanchflower *et al.*, 1993b). The development of biosensor technology has created other screening methods that are both rapid and reusable. This technology has already been applied as an alternative screening method to ELISA for sulphonamides in pigs both in the laboratory (Crooks *et al.*, 1998a) and on-site at an abattoir (Baxter *et al.*, 1999). A multi-residue method has also been published using an adapted sensor (Crooks *et al.*, 2001). This technology could also lead to the development of procedures that could generate real-time results without the need for time consuming clean-up steps and deconjugation of analytes, as has already been demonstrated with salbutamol (Elliott *et al.*, 1998).

1.9.2. Confirmatory methods

After a positive screening test - unambiguous detection of a compound in the sample is required to confirm its presence and quantify it. EU legislation states:

"Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods." (European Commission, 2002)

This has led to mass spectrometry becoming the method of choice, though chromatography linked to other methods such as fluorescence, diode-array detection and full scan UV/Vis detection are also suitable. Two types of Mass Spectrometry (MS) are frequently employed: Gas Chromatography (GC) and liquid chromatography (LC). GC-MS is generally used for thermally stable, non-polar and volatile compounds. Many

compounds cannot be analysed, as they become unstable due to the high gas, column, and MS source temperatures. Less volatile analytes can be derivatised to allow its application. Compounds such as steroids can be routinely screened and confirmed using this method as they are thermally stable, only require a simple extraction and can easily be derivatised (Blanchflower *et al.*, 1993b). LC-MS has no such disadvantages as all separations are carried out at ambient temperature. Such separation depends on a number of factors - the analyte solubility in mobile phase, chemical functionality of the analyte, pK_a value of the analyte, HPLC column properties, and the mobile phase. The mobile phase may be tailored to one or a mixture of several different analytes to allow specific separations and/or to increase or decrease their retention time changing the run time per sample. Antibiotics are generally insufficiently volatile, polar or thermally unstable to allow GC-MS. Two examples are tetracyclines, which lack the volatility for GC-MS, and aminoglycosides that are basic, highly hydrophilic, and thermally labile. Therefore, LC-MS has become the preferred method of analysis for such compounds.

1.10. Liquid chromatography – mass spectrometry

HPLC can be linked to MS through various types of interfaces. The separation capabilities of HPLC therefore can be linked to the specificity of mass spectrometry, allowing polar, thermally labile or non-volatile compounds to be analysed that are not always amenable to GC-MS. Several types of interface between the HPLC system and the mass spectrometer have been developed over the last 20 years. The most widely used are thermospray, Atmospheric Pressure Chemical Ionisation (APCI), and electrospray. Electrospray has become the increasing popular method in recent years, not only in the field of antibiotics and veterinary drugs, but also in other areas with applications such as peptide and nucleotide characterisation etc. Since 1992 it has dominated the annual American Society

for Mass Spectrometry (ASMS) Conference on Mass Spectrometry and Allied Topics (Niessen, 1998). This more than likely goes hand in hand with increasing commercial availability and application.

1.10.1. Electrospray

Electrospray like APCI occurs under atmospheric pressure. The HPLC eluate is nebulized into electrically charged droplets, from which ions are liberated by ionisation at atmospheric pressure and introduced to the mass spectrometer under vacuum. In positive mode, the mobile phase eluate passes through a capillary tube that has a high negative electric field applied at the tip, pulling the positively charged ions in the liquid towards it. The negatively charged ions discharge against the wall of the capillary tube becoming neutral. Small electrically charged droplets leave the surface of the mobile phase when their electrostatic charge becomes stronger than the surface tension creating a spray. After leaving the tip, the solvent in each droplet evaporates creating many smaller daughter droplets and a finer spray. These are attracted towards an oppositely charged plate and taken into the mass spectrometer under vacuum. When negative ionisation is required, the power supply is simply reversed. The capillary tube tip becomes negatively charged, the positive ions discharge against the capillary wall leaving only the negative ions to aerosol and move towards the positively charged base plate. Like APCI, a high velocity sheath is used allowing the handling of solutions that are more aqueous and higher mobile phase flow rates – up to 2 ml min $^{-1}$. This is known as 'ionspray' and is commonly incorporated into most commercial instruments. Electrospray makes use of the ions present in the solution due to evaporation of solvent. The addition of strong acids, for example acetic and formic acid at low concentration, or adjustment in pH can greatly help with the protonation of polar samples and improve sensitivity for mass spectral analysis in positive

mode. Triethylamine can serve the same function in negative mode. As with APCI, electrospray can also provide CID to increase the number of molecular fragments if necessary.

1.10.2. Liquid chromatography – mass spectrometry considerations

Though the analytes in question may be separated satisfactorily using HPLC connected to a UV/Vis detector, such a method cannot always be directly transferred for use in confirmation by substitution of detection type. The use of phosphate buffers and ion-pairing agents is common in HPLC. Such compounds are too involatile to allow use with the interfaces commonly used in modern LC-MS. The involatile ions in the buffer can build up as salt depositions at the tip of the LC interface blocking the flow of the mobile phase after a few samples. These buffer ions can also actively suppress ionisation of the analyte, decreasing the sensitivity of the method. The development of many new HPLC stationary phases, the use of low flow rate narrow bore columns, and the substitution of more volatile buffers, allow these problems to be overcome. The organic constituents of the mobile phase can also be changed - not only to allow different separation but also to enhance the sensitivity of the mass spectrometer. The simple change of the organic phase from acetonitrile to methanol has been noted in the laboratory to dramatically increase the sensitivity of many methods. The addition of 0.1 – 0.2 % acid in the mobile phase such as formic and acetic have also been used to help enhance the ionisation of analytes, improve peak shape and increase sensitivity in positive mode. However, such effects are not seen in the opposite mode as the positive charge of the acid affect ionisation. Care must be taken in the selection of such agents to improve peak shape and sensitivity as they may linger in the source of the MS and directly affect the measurement of other analytes. For example, if analysis is performed in negative mode after the use of Trifluoroacetic acid (TFA) as an

ionisation enhancer, the method sensitivity can be compromised, as the TFA will mop up the negative ions formed upon ionisation. Such effects have been seen in this laboratory in the determination of chloramphenicol after using a mobile containing TFA.

The mass spectrometer can be set to detect the molecular and/or fragment ions of the parent compound. The chromatographic separation before analysis plays a large part in partitioning interferants of the same molecular weights from the analyte in question. These considerations in many methods allow simple clean-up and extraction steps due to the degree of selectivity and sensitivity that LC-MS analysis can give. Modern mass spectrometers are capable of simultaneously measuring specific separate molecular masses with a very short dwell time on each. However, effects from the sample matrix that has been carried through the method exist. Ion enhancement, ion suppression, and biotransformation of the analyte are all effects encountered even with the selectivity possible with modern LC-MS.

Ion enhancement and suppression are the increase or decrease, respectively, of the mass spectrometer signal for an analyte due to the composition of the matrix carried over from the sample. These can usually be compensated for by the addition of an appropriate internal standard at the beginning of sample extraction. The internal standard is preferably a deuterated form of the analyte to be measured, exhibiting the same chromatography and fragmentation properties as the analyte, but with a molecular weight, several mass units higher, that allows differentiation by the mass spectrometer. The internal standard will compensate for any losses in extraction of the analyte, as well as for ion enhancement or suppression that the analyte undergoes – increasing the accuracy of the method. If an internal standard is unavailable, it is common in multi-residue methods to link the

measurement of an analyte to the internal standard of another similar compound. For example, Ferchaud *et al.* (1998) reported a method for the analysis of testosterone and several of its metabolites using d_3-methyltestosterone as the single internal standard. Alternatively, if no internal standards are available, standards within each run may be dissolved in blank extracted matrix to allow for any matrix-related effects on the signal. However, this has the disadvantage of increasing the number of samples to be put through extraction procedures per run, and will limit the number of unknown samples that can be analysed in each run.

1.10.3. Application of LC-MS to the analysis of veterinary drug residues in Northern Ireland

LC-MS has been in use as a confirmatory residue method within Veterinary Sciences Division (VSD) for more than 10 years. Various methods have been developed and published, using a range of LC-MS techniques. Such methods have been applied to the statutory testing of samples taken under the National Surveillance Scheme for NI. Some examples have been outlined from the first use of thermospray MS in the late 1980s to electrospray MS-MS in the present day. One of the earliest thermospray-LC-MS methods to be reported from this laboratory was in 1989 and was used for the rapid screening and quantification of clenbuterol residues in bovine urine (Blanchflower and Kennedy, 1989b). This was closely followed by thermospray methods for the determination in tissue of the anthelmintic, nitroxynil (Blanchflower and Kennedy, 1989a), and rafoxanide (Blanchflower *et al.*, 1990). Blanchflower *et al.* (1993a) published a method for the determination of chloramphenicol in plasma, milk, and tissue using deuterated chloramphenicol as an internal standard. This method used piperonyl butoxide in ethyl acetate during tissue homogenisation to inhibit *in vitro* metabolism of chloramphenicol.

Analysis was carried out by HPLC linked via a thermospray interface to a Vestec MS system. This was followed by a new multi-residue method for the determination of penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin in milk, kidney and muscle samples (Blanchflower *et al.*, 1994). The samples were subject to a simple liquid/liquid extraction and analysis by HPLC connected by an electrospray interface to a VG Platform MS, operating in negative mode.

McCracken *et al.* (1995) published a method for the detection of the unstable and rapidly metabolised antimicrobial, furazolidone using thermospray MS. The assay was used to demonstrate the instability of furazolidone residues both *in vitro* and *in vivo* that makes such direct detection difficult. This work was subsequently followed up with a method for the determination of the protein-bound marker residue of furazolidone - AOZ. In this method, AOZ was converted into a nitrobenzaldehyde derivative, NPAOZ, which was detected using thermospray MS after HPLC separation on a C_{18} column (McCracken and Kennedy, 1997). This method allowed further studies on the pharmacokinetics and bioavailability of furazolidone in pigs (McCracken *et al.*, 1997) and in eggs (McCracken *et al.*, 2001). Through the analysis of AOZ in pigs, it has been shown that it is possible to pass AOZ from medicated to unmedicated animals after use of the same housing, thus giving rise to the possibility of false 'positive' results (McCracken *et al.*, 2000).

Nitroimidazoles are bactericidal drugs that have been used as a broad-spectrum antibiotic in the poultry and cattle industries, but are now banned from use within the EU. Few methods have been published for the analysis of these compounds. Cannavan and Kennedy (1997) published a method for the determination of dimetridazole (DMZ) in both poultry tissue and eggs, using thermospray MS in positive mode. An internal standard

(deuterated DMZ) was employed to increase accuracy by compensating for losses during extraction. This method was used to investigate the effects of contamination levels of DMZ in feed on chickens and the possible occurrence of violative residues.

Cannavan *et al.* (1999) published an HPLC electrospray MS method for the determination of nicarbazin in animal feedingstuffs. This was followed by a method for the quantitative determination of 4,4-dinitrocarbanilide (DNC) the marker residue for the use of the drug in chicken liver and eggs. This method makes use of the MS-MS capabilities of the laboratory. After extraction, analysis was carried out using an isocratic mobile phase of 0.05 mol l^{-1} ammonium acetate in acetonitrile-water (75 : 25 v/v) on a Luna C_{18} column connected to a Micromass Quattro MS-MS spectrometer in negative mode (Yakkundi *et al.*, 2001).

MS-MS linked to HPLC gives enhanced sensitivity and selectivity to the methods and has recently allowed the development of a method for the confirmation of halofuginone, a prophylactic against coccidiosis used in commercial poultry production (Yakkundi *et al.*, 2003). This has led to the study of the pharmacokinetics of such residues in chicken liver and eggs due to contamination levels in feed (Yakkundi *et al.*, 2002).

1.11. European Union legislation concerning LC-MS method validation

Before a method can be submitted for both internal and official regulatory approval, it must undergo systematic validation. The goal of validation is to determine the limits of variability for the method over the concentration range it is to be applied. Current criteria for method validation are covered by EU Commission Decision 2002/657/EC (European Commission, 2002). These contain specific validation details for LC-MS and MS-MS

techniques and guidelines for the determination of method performance characteristics. The EU defines validation for LC-MS confirmatory methods in several parts: compound identification, recovery, repeatability, within laboratory reproducibility, reproducibility, the decision limit (CCα), and detection capability (CCβ).

1.11.1. Compound identification

Criteria for chromatographic and analyte identification must be applied to confirm the presence of a specific compound when using a LC-MS method:

Chromatographic criteria

The minimum acceptable retention time for any analyte to be validated must be twice the void volume of the column. When no internal standard is available, the retention time of the analyte should closely match that of the same analyte in a corresponding standard. A margin of error of ± 5% of the retention time is allowed for this. The peak width at half the maximum height also should be within the 90 – 110% range of the original width.

When an internal standard is available the ratio of the retention time of the analyte to that of the standard must fall within predefined limits. EU legislation defined this as:

"The ratio of the chromatographic retention time of the analyte to that of the internal standard i.e. the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 0,5 % for GC and ± 2,5 % for LC." (European Commission, 2002)

Analyte identification criteria

All detected ions should be expressed as a percentage of the intensity of the most intense ion or transition product, known as the relative ion intensity. In all samples, ion intensities must correspond to those of the standards or spiked samples at comparable concentrations within predefined limits. These maximum permitted tolerances are outlined in Table 1.1.

Table 1.1. Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques.

Relative intensity (% of base peak)	EI-GC-MS (Relative)	CI-GC-MS, GC-MS-MS, LC-MS, LC-MS-MS (relative)
> 50 %	± 10 %	± 20 %
> 20 % - 50 %	± 15 %	± 25 %
> 10 % - 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

(European Commission, 2002)

For confirmatory methods, a minimum of one relative ion intensity should be measured. A system of Identification Points (IP) is also awarded for each analyte ion identified based on the type of MS used. The number of points awarded per ion reflects the ability of an analyser to specifically select the analyte molecule or mass fragment e.g. tandem MS

fragments will be awarded more IPs than single MS. The IP assignment system is outlined in Table 1.2.

Table 1.2. The relationship between a range of MS technique and Identification Points earned.

MS technique	Identification points earned per ion
Low resolution mass spectrometry (LR-MS)	1.0
LR-MS Precursor ion	1.0
LR-MS Transition products	1.5
High resolution mass spectrometry (HR-MS)	2.0
HR-MS Precursor ion	2.0
HR-MS Transition products	2.5

(European Commission, 2002)

Transition products can include both daughter and granddaughter products of the monitored precursor ion. Therefore methods using MS-MS will score 1.5 IPs for each ion measured, along with an additional 1 IP for the precursor ion whether it is measured or not. For substances having an anabolic effect and unauthorised substances (Group A of Annex I of Council Directive 96/23/EC), a minimum of 4 IPs are required for confirmatory methods. However, for veterinary drugs, including unlicensed substances used for veterinary purposes and contaminants (Group B of Annex I of Council Directive

96/23/EC), only a minimum of 3 IPs are required. Up to three separate techniques may be used to achieve the minimum number of IPs required.

1.11.2. Recovery

Recovery is the percentage of the true concentration of a substance recovered during the analytical procedure. When there is no certified reference material available, recovery is assessed using fortified blank matrix samples. Six aliquots of blank matrix are fortified at each of 1, 1.5 and 2 times the Minimum Required Performance Limit (MRPL) for banned compounds or at 0.5, 1 and 2 times the MRL for legal compounds. From these the percentage recovery for each sample and mean recovery for each level of fortification can be calculated.

1.11.3. Repeatability

This is the degree of agreement among results at 1, 1.5 and 2 times the MRPL or at 0.5, 1 and 2 times the MRL and can be assessed in the same analytical run as recovery. The mean concentration, Standard Deviation (SD), and Coefficient of Variation (CV) are calculated for each level of fortification.

1.11.4. Within-laboratory reproducibility

This is the degree of agreement among different sets of results when the procedure is repeatedly assessed at the same fortified concentration, over three separate runs, on separate days, to allow for different environmental conditions, analytical variations, operators, etc. This value is again is expressed as the mean concentration, SD and CV for each fortification level.

1.11.5. Decision Limit (CCα)

EU legislation defines this for banned compounds and substances without a permitted residue limit as:

"The limit at and above which it can be concluded with an error probability of α that a sample is non-compliant." (European Commission, 2002)

CCα, the decision limit, is the limit at which it can be decided that a sample is truly violative (greater than zero for an unauthorised substance) with an error probability of α. This study only involved compounds that have been banned within the EU, therefore α = 1%.

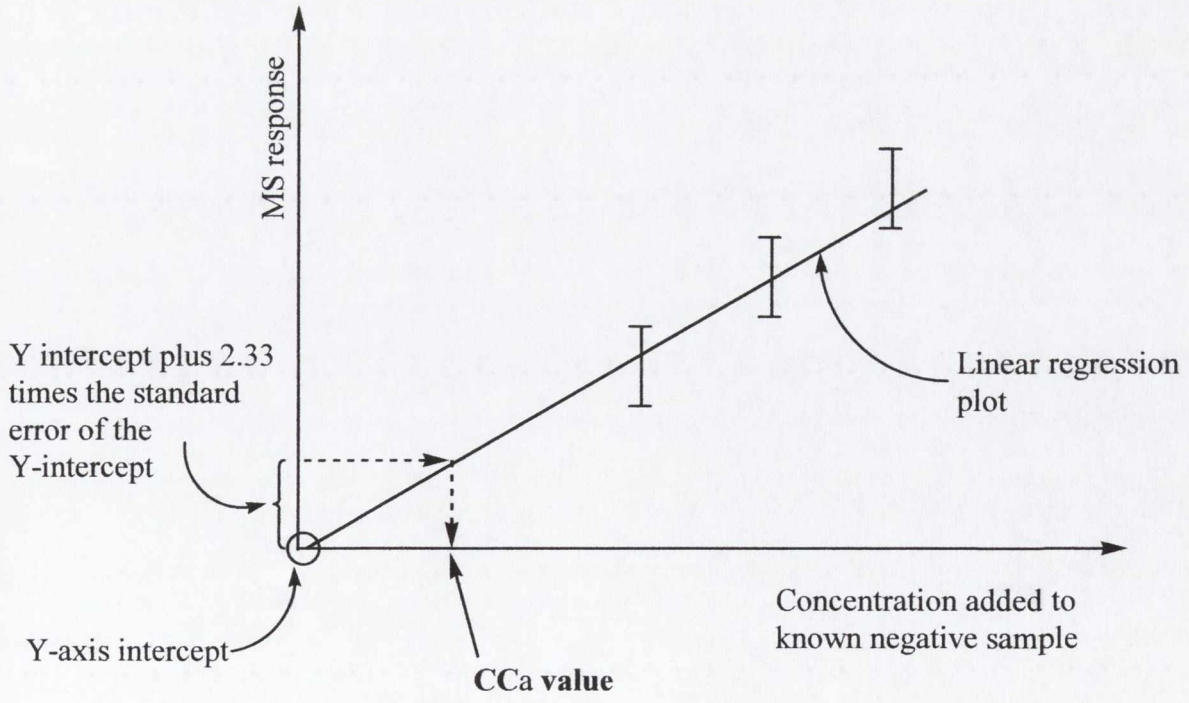

Fig. 1.1. CCα and Y intercept

Representative blank material should be fortified at below the MRPL (if assigned), analysed and the MS response versus fortified concentration plotted and the Y-axis intercept calculated. CCα is calculated from the graph as the concentration corresponding to 2.33 times the standard error of the Y intercept added to the Y intercept.

1.11.6 Detection Capability (CCβ)

EU legislation defines this for banned compounds and substances without a permitted residue limit as:

"The smallest content of a substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – β." (European Commission, 2002)

CCβ, the detection capability, is the smallest content of analyte that may be detected, identified, and quantified in a sample with an error probability of β. In the case of unauthorised substances, the β error should be less than or equal to 5%. The CCβ can be extrapolated graphically through the addition of 1.64 times the standard error of the Y intercept to the Y value for mean measured content at the decision limit (CCα).

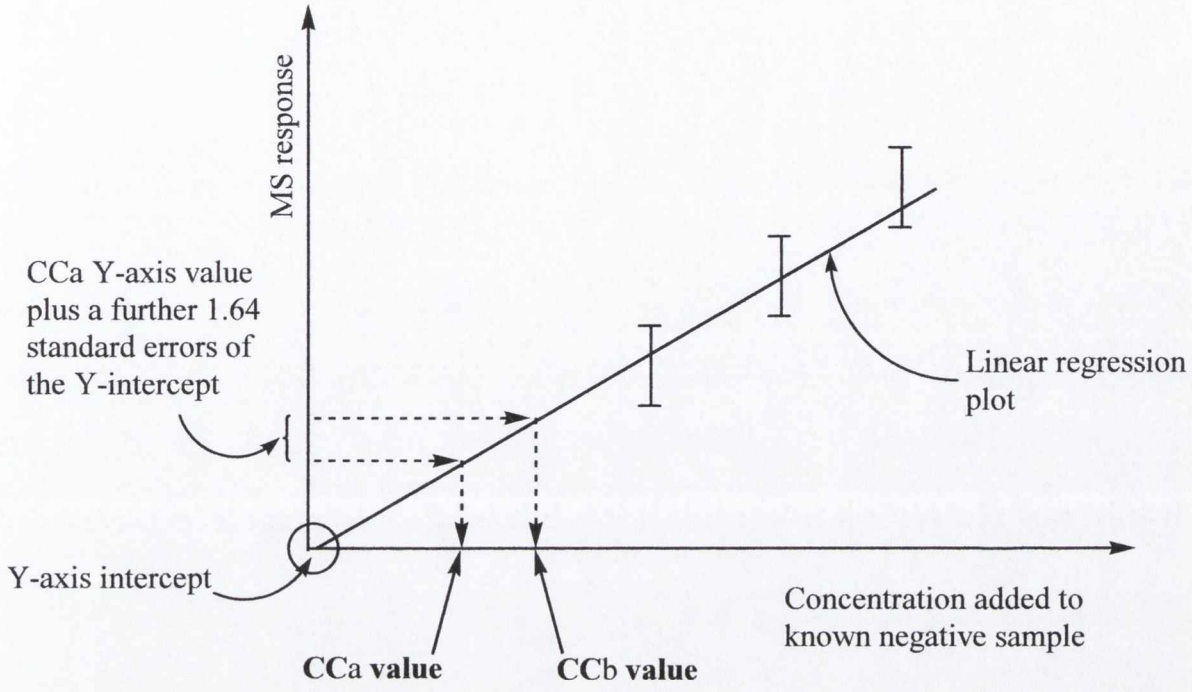

Fig. 1.2. CCα and CCβ graph

1.12. Quinoxaline-N-dioxides

The quinoxaline-N-dioxides are yellow crystalline solids with melting points > 200 °C and their solubility in water and other organic solvents is dependent on the side chain present. They were first prepared as a potential (and undemonstrated) antagonist to vitamin K activity and later discovered to have antimicrobial properties. Quinoxaline-N-dioxides are classed as 'other antibacterial drugs' due to their structure, which does not easily fit into any of the major classes of antibacterial agents. Structurally they are derivatives of the quinoxaline ring system. Introduction of different sidechains (-R) on the 2nd and 3rd carbon in the quinoxaline-N^{1},N^{4}-dioxide ring gives rise to a small number of compounds which all exhibit anti-bacterial and coccidiostatic properties (see Fig. 1.3.). A few of these compounds have additionally exhibited growth-promoting effects if used as an infeed additive, several already having been used within the livestock industry. Quindoxin (QDN) was added at 50 mg kg^{-1} infeed as a growth promotant in young chickens within

the poultry industry (Broome and Bowie, 1972). Carbadox (CBX) and Olaquindox (OQX) were used as prophylactic agents and growth promotants in young pigs at concentrations in feed of 50 and 100 mg kg^{-1}, respectively (see Section 1.13.4. and Section 1.14.4.).

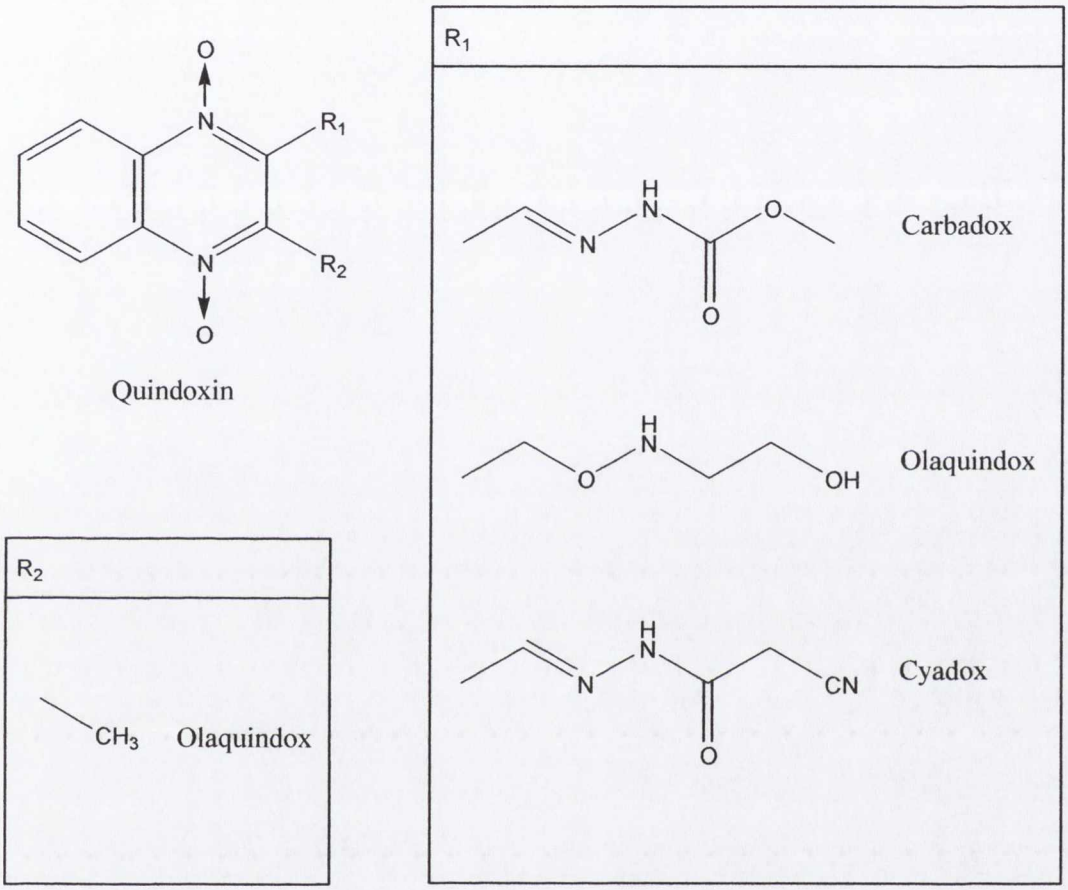

Fig. 1.3 Structures of QDN and its derivatives, CBX, OQX and cyadox.

1.13. Carbadox

1.13.1. Chemistry and physical properties

CBX, methyl-3-(2-quinoxalinyl-methylene)carbazate-N^1,N^4-dioxide or 2-formylquinoxaline-1,4-dioxide carbomethoxyhydrazone, has a molecular formula of

$C_{11}H_{10}N_4O_4$ and a molecular weight of 262.23 (see Fig. 1.4.), and has a melting point of 239.5 – 240 °C.

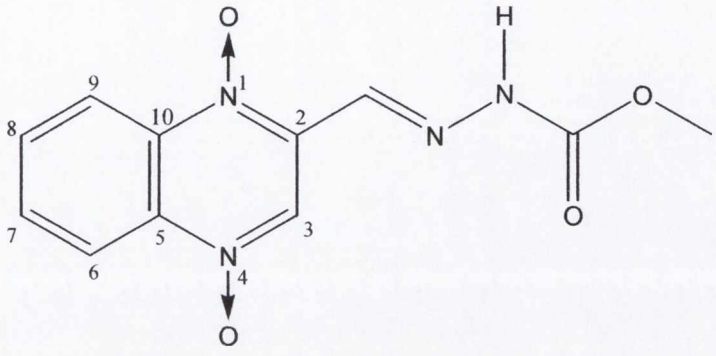

Fig. 1.4. Chemical structure of CBX.

1.13.2. Absorption, metabolism and pharmacokinetics

Shortly after the start of oral administration (3-5 hours) of CBX, several metabolites have been identified in plasma: CBX, desoxycarbadox (desoxy-CBX), carbadoxaldehyde, and Quinoxaline-2-Carboxylic Acid (QCA). After 24 hours another metabolite - methylcarbazate was identified in plasma along with trace amounts of hydrazine (WHO/FAO, 1991). Upon withdrawal of medication, CBX metabolites have been reported to disappear rapidly from serum and blood within 48 to 72 hours. Disappearance from tissues such as muscle, liver and kidney is seen on a similar time scale, with the desoxy-CBX metabolite persisting for slightly longer (MacIntosh *et al.*, 1985). Twenty four hours after the withdrawal of medication, the major detectable residue is QCA along with only small amounts of methylcarbazate, CBX and desoxy-CBX (Lauridsen *et al.*, 1988). The enzymes involved in CBX catabolism have not yet been elucidated. Once CBX and desoxy-CBX are converted to QCA no further metabolism of the drug occurs (see Fig. 1.5.).

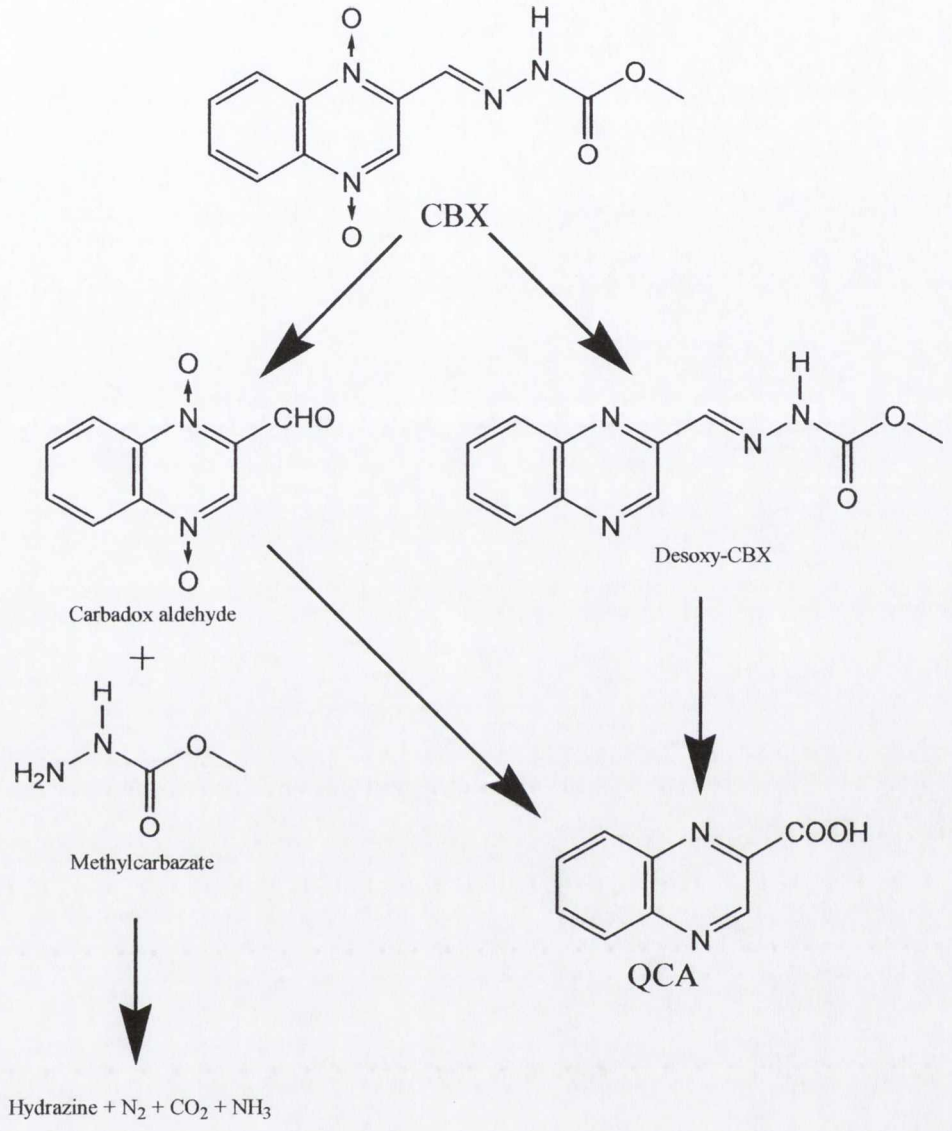

Fig. 1.5. Simplified pathway for the breakdown of CBX.

The majority of residues are removed from the system within 72 hours of medication. The major pathway for excretion is via the urine (60-70%) with lesser amounts (10-20%) in faecal matter carried by bile (WHO/FAO, 1991). The QCA metabolite may be present in tissues for extended periods of time, with the exception of muscle where elimination occurs within the normal 72 hours (Baars *et al.*, 1990). Using pigs given rations medicated at 20 mg kg^{-1}, which is less than half the prior recommended prophylactic level, QCA

residues were present in liver samples 30 days after the cessation of treatment (Lauridsen *et al.*, 1988). Another study using the recommended prophylactic concentration (50 mg kg^{-1}) found that QCA residues were still detectable in liver, 77 days after the cessation of treatment (Rutalj *et al.*, 1996).

1.13.3. Growth promoting action

CBX and other QDN derivatives have been shown to be effective growth promotants for young pigs by administration in feed (Thrasher *et al.*, 1969). Significantly increasing the weight gains, feed efficiency and reducing the rearing period for young pigs even on protein poor diets (Bourdon *et al.*, 1977). Early in the use of antimicrobials, the mode of action of growth promotion was thought to be related to effects on gastrointestinal microflora (Visck, 1978). Even today, this mode of action is speculative, the theory based on the suppression of catabolism of urea and amino acids and decreased carbohydrate fermentation by the action of the antibacterial agent on gut flora. This leads to the increased availability of nutrients and amino acids to the animal host. Initially this was suspected of CBX (Gedek, 1979). However, in pigs fed twice the recommended prophylactic dose (100 mg kg^{-1}) severe adrenocortical damage and an enlarged glomerular zone was discovered upon autopsy (Van der Molan *et al.*, 1985). Hypoaldosteronsium i.e. a reduction in aldosterone production resulting in the loss of sodium and potassium resulting in hypotension, was also reported with CBX use *in vitro* (Spierenburg *et al.*, 1988b) and *in vivo* (Van der Molan *et al.*, 1986). Hence, CBX seems to have very specific effects on adrenal tissue in pigs. This has led to the discovery that CBX has anti-catabolic effects through reduced secretion of cortisol, corticosterone, aldosterone and deoxycortisol *in vitro* and *in vivo* by porcine adrenocortical cells (Jager *et al.*, 1996). In addition to a reduction in the cortisol-derived hormones, CBX increases the rate of secretion of

progesterone. This implies that CBX irreversibly or non-competitively inhibits the 21β-hydroxylase enzyme responsible for the conversion of progesterone to deoxycorticosterone and 17-OH-progesterone to deoxycortisol (see Fig. 1.6.). These are steps in the major metabolic pathways for the production of aldosterone and cortisol (Jager *et al.*, 1996).

Progesterone is a precursor in the anabolic pathway for the testosterone, and increased secretion of progesterone will lead to increased secretion and plasma levels of testosterone. Testosterone is known to have many anabolic effects and in conjunction with a high protein food intake (such as in pig rations) will give a positive nitrogen balance in the body through the increased uptake and retention of dietary nitrogen. This ultimately leads to amplified muscle and adipose tissue formation. This is supported by CBX being noted as having a marked effect on the uptake and retention of dietary nitrogen (Yen *et al.*, 1976).

CBX is rapidly metabolised to desoxy-CBX that has been found to have identical inhibitory effects (Jager *et al.*, 1996). Consequently, though CBX has a direct anabolic effect, it also acts as a pro-drug extending the therapeutically active time through its metabolite. CBX does not overly affect the secretion of the other major anabolic hormones – progesterone, HO-progesterone, androstenedione or dehydro-epi-androsterone (Jager *et al.*, 1996). Normal serum levels of each will have enhanced effects as secretion of the opposing negative feedback catabolic hormones is reduced. CBX has also been shown to inhibit the 7α-hydroxylase enzyme for the conversion of cholesterol to 7α-hydroxycholesterol, the first step in the catabolism of steroids and the production of the bile acids (Tracy and Jensen, 1987).

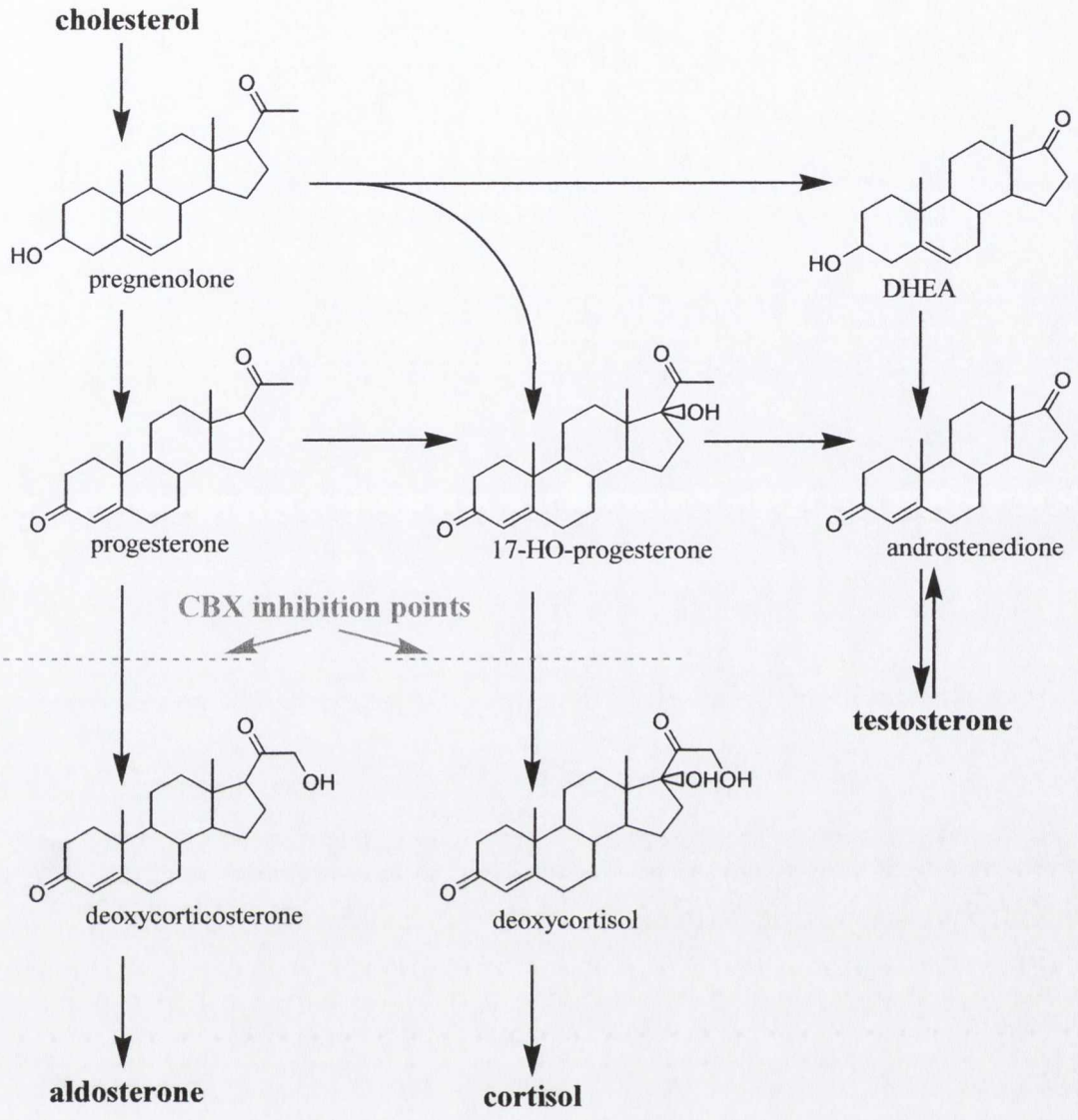

Fig. 1.6. CBX inhibition points in the major metabolic pathways for the biosynthesis of adrenocorticosteriods.

This will lead to increased steroid production (and ultimately progesterone secretion, through the previously mentioned inhibited enzymes), as more precursor molecules are available – again enhancing the effect of CBX on the anabolic system. Steady state kinetic studies with CBX in weaned piglets feed medicated feed (50 mg kg^{-1}) over 90 days showed CBX to maintain steady concentrations in porcine blood between 10 – 50 ng ml^{-1} (De Graaf *et al.*, 1988). With the daily administration of medication, continuous anabolic

effects are exerted on the porcine system. Such effects will persist for some time after the cessation of treatment, as time is required to reverse the inhibition of aldosterone production.

1.13.4. Veterinary use and clinical symptoms

Though primary use has been as a growth promoter in both piglets (Jost, 1979) and broiler chickens (Schüler *et al.*, 1977), CBX also has a clinical function with antimicrobial effects on gram-negative bacteria. Combined with pryantel tartrate in feeds it acted as a strong prophylactic against swine kidneyworm (Stewart *et al.*, 1979) and was used in the treatment of infectious atrophic rhinitis (Farrington and Shively, 1978). Action in the prevention of enteric colibacillosis (Pujin *et al.*, 1974) and reduction in the severity of salmonellosis (Troutt *et al.*, 1974) were also reported. The main clinical use of CBX was as a prophylactic antitreponemal agent. Use of CBX medicated feed (50 mg kg^{-1}) prevented the clinical signs of swine dysentery (haemorrhagic enteritis), a disease commonly found in the pig industry (Williams and Shively, 1978). Prevention was through bacteriostatic action on the growth of *Treponema hydysenteriae* (Williams and Babcock, 1976), the primary pathogen in swine dysentery (Hughes, 1975). Pharmacokinetic studies have shown that the concentrations of CBX in the gastrointestinal tract after medication (50 mg kg^{-1}) are high enough to have a bacteriostatic action. Consequently oral dosing was an effective prophylactic treatment against invading treponemes (De Graaf *et al.*, 1988).

Feed concentrations of 100 – 150 mg kg^{-1} have also been used as a direct treatment for *Treponema hydysenteriae* infection. Conversely at such elevated medication levels CBX use causes decreased secretion of aldosterone, leading to induced hypoaldosteronism in

severe cases (Van der Molen *et al.*, 1989b) – questioning its efficiency as a direct treatment, but not as a prophylactic. Extended use (>5-7 days) at such concentrations (100 - 150 mg kg^{-1}), can become detrimental to animal health. Induced symptoms include dehydration, reluctance to eat, loss of condition, posterior paresis, greatly increased heart rate, decreased appetite, wall licking, urine drinking and an appetite for salt, the passing of hard faecal matter and ultimately death (Nabuurs and Van der Molan, 1989; Van der Molan, 1988). Such clinical symptoms can be attributed to the secondary effects of CBX on the renin-angiotensin system (RAS) and by the inhibition of bile acid synthesis.

The inability to produce aldosterone in turn leads to hypoatraemia and hyperalaemia and a decreased plasma ratio of sodium to potassium – giving a negative water balance, decreased plasma volume, an increased heart rate and dehydration (Power *et al.*, 1989). Through the negative feedback mechanism between the RAS and aldosterone plasma levels, decreased aldosterone production causes the rise of plasma angiotensin II levels (Van der Molan *et al.*, 1989a) to trigger its production and secretion from the adrenal glomerulosa. Pigs with CBX intoxication have been reported to show clinical symptoms such as depraved appetite, wall licking, urine drinking (appetite for salt) even after medication has ceased and aldosterone levels have returned to normal (Nabuurs and Van der Molan, 1989). These might be explained by the continual increased RAS activation through angiotensin II, which has been shown responsible for salt appetite. CBX has been reported to inhibit the 7α-hydroxylase enzyme in the metabolism pathway of cholesterol to 7α-hydroxycholesterol (Tracy and Jensen, 1987). This is a rate limiting enzyme for the biosynthesis of the bile acids (lithocholic acid, chenodexoycholic acid, and hydrodeoxycholic acid) in pigs. Continual elevated levels of CBX in the blood will

severely impede the production of bile acids and therefore greatly reduce biliary secretion into the gastrointestinal tract *via* the bile duct, leading to the passing of hard faecal matter.

1.13.5. Mutagenic properties and antibacterial activity

Since the introduction of CBX as an antimicrobial agent, many studies have shown that it also exhibited mutagenic properties. Other coccidiostatic agents such as dimetridazole and ronidazole have been shown to increase the mutation rate in *Klebsiella pneumoniae*, *Escherichia coli* and *Citrobacter freundii* using fluctuation assays (Voogh *et al.*, 1974). Such suspicions led to the investigation of the possible mutagenic properties of CBX. Early in its use as a growth promoter CBX was shown to cause *in vitro* chromatid aberrations in bone marrow cells and increased micronucleated polychromatic erythrocytes - an indicator of bone cell genetic aberrations (Oud *et al.*, 1979). *In vitro* mutagenic changes were also reported with *Bacillus subtilis* and *Salmonella typhimurium* (Yoshimura *et al.*, 1981). CBX has been shown as mutagenic at very low concentrations ($2.0 - 500 \times 10^{-5}$ mM). Using the Ames test with *Salmonella* spp. such action has been demonstrated to be without metabolic activation (Voogh *et al.*, 1980) i.e. the compound itself is the mutagen.

With *in vitro* mutagenic activity demonstrated, establishing the effect of the drug *in vivo* in mammals was of importance as personnel within the agricultural industry would be at risk of exposure. CBX use *in vivo* increased the number of micronucleated polychromatic erythrocytes in rats (Cihák *et al.*, 1983) and demonstrated aberrant effects in the foetal liver of pregnant mice (Cihák and Vontorková, 1985). Short-term teratogenic and embyrolethal effects have been confirmed *in utero* in rats (Yoshimura, 2002). The above

studies show that CBX is mutagenic and carcinogenic *in vitro* and carcinogenic, genotoxic and embyrolethal *in vivo*.

Positive results have been published for CBX using the *rec* test that assays UV-induced-like damage and single strand breaks in cellular DNA (Yoshimura *et al.*, 1981). Hence, CBX directly attacks bacterial DNA, leading to errors in the DNA template and causing both base-pair substitutions and frame-shift mutations because of errors in the DNA repair process. This will subsequently inhibit the synthesis of DNA i.e. a bacteriostatic effect. Such conclusions are supported by reports that DNA repair enzyme deficient spp. are more susceptible to CBX and other quinoxaline-N-dioxides (Beutin *et al.*, 1981; Suter *et al.*, 1978).

The endogenous mutagenicity of the parent compound and ring metabolites has been demonstrated to be linked to the presence of the N-oxide groups of the quinoxaline ring, as quinoxaline-N-oxide exhibits lower mutagenic properties than the N,N-dioxide form (Beutin *et al.*, 1981). The reduced metabolite desoxy-CBX has also been shown to be a potent hepatocarcinogen and tumour inducing agent *in vivo* in rats and mutagenic in cell tests *in vitro* (WHO/FAO, 1991), however the mechanism of this action is currently unknown. During metabolism of desoxy-CBX the side chain attached to the 2^{nd} carbon of the quinoxaline ring is cleaved off, giving a methylcarbazate group that can be further broken down to hydrazine. Many unrelated studies with hydrazine have shown that it is a genotoxin and tumour-inducing agent both *in vitro* and *in vivo* (WHO/FAO, 1991). Hydrazine is a minor metabolite and would only be expected to be present *in vivo* for a short period; whether such trace levels will have any mutagenic effect has yet to be

clarified. Only the QCA metabolite has been shown not to exhibit any mutagenic properties (Yoshimura *et al.*, 1981).

The antibacterial activity and the mutagenicity of quinoxaline-N-oxides are suspected of being linked. Both activities are enhanced under anaerobic conditions, suggesting that each are due to the same activation mechanism (Beutin *et al.*, 1981) i.e. the reduction, alteration or aberration of DNA through the N-oxide groups on the quinoxaline ring. This has been supported by cyclic voltammetry data, which suggests that some relationship exists between the reduction potential of the compounds and their antimicrobial activity (Crawford *et al.*, 1986).

1.13.6. European Union legislation concerning carbadox

CBX was first listed as a zootechnical feed additive under Part E (other additives) of the Annexes to EU Council Directive 70/524/EEC in 1974 by Council Directive 74/378/EEC. EU authorisation was granted for its use as a feed additive in pigs in 1987 at medication concentrations of 50 mg kg^{-1} by the amendment and addition of CBX to Annex I of the same Council Directive (European Commission, 1987a). The conditions of its use were further amended in 1990 (European Commission, 1990c) and 1996 (European Commission, 1996a). Authorisation was granted in 1987 despite worldwide reports in 1972, 1984 and 1985 that CBX and desoxy-CBX were suspected carcinogens and mutagens.

During the 1980s and early 90s when CBX was available as a pre-mix formulation under licence in the EU, several reports were published that CBX caused genotoxicity both *in vitro* and *in vivo*. Desoxy-CBX and the side-chain metabolite methyl carbazate (that can

be further metabolised to generate hydrazine) were also suspected carcinogenic and tumour inducing agents (see Section 1.13.5.). The Federal Republic of Germany requested on 14th February, 1997, a re-evaluation of quinoxaline-N-dioxides, due to concerns over possible risks to consumers, operators and animals. Both SCAN and the European Commission acknowledged that CBX was carcinogenic and genotoxic in rodents, though SCAN noted that quinoxaline-N-dioxides were acceptable for use under their previously defined conditions (SCAN, 1998). The European Commission believed that it was not possible to set a threshold limit for a genotoxic agent, when even small quantities can induce tumour-initiating mutations, consequently not allowing withdrawal times to be set. CBX was withdrawn from use in the EU on 1st January, 1999, under Commission Regulation No 2788/98 (European Commission, 1998), over these mutagenic concerns and the unacceptable risks it posed to the consumers, and industry and farmer workers. CBX was freely available in the Republic of Ireland up until the 1st September 1999 (though it had not been available in the UK since the mid. 1980s). It is still available as a medicated feed product in the USA, Canada and the rest of the world under licence. CBX does not come under EU Council Directive No 2377/90, as it is not considered a veterinary medicine and was only ever licensed as a zootechnical feed additive. Therefore, it has been removed from Annex B of EU Council Directive 70/524/EEC that governs the use of zootechnical feed additives, but not added by amendment to Annex IV of EU Council Directive No 2377/90. The current EU prohibition on CBX use is enforced under the UK National Surveillance Scheme by monitoring for the presence of the marker residue, QCA, in livestock at slaughter.

1.13.7. Quinoxaline-2-carboxylic acid

Quinoxaline-2-carboxylic acid, which has the molecular formula $C_9H_6N_2O_2$, has a m.w. of 174.158 and is soluble in alcohols and slightly soluble in water. It is a quinoxaline ring with a carboxylic acid group attached to the 2nd carbon of the ring (see Fig. 1.7.). Unlike its precursors, there are no datively bonded oxygens attached to the nitrogen atoms on the ring.

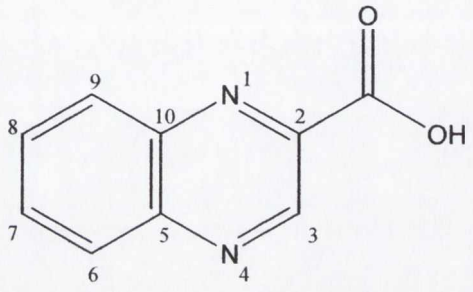

Fig. 1.7. Chemical structure of QCA.

QCA has been assigned as the marker residue for CBX, as it is deemed the most persistent measurable metabolite through metabolism and depletion studies (Ferrando *et al.*, 1975). The target tissue of this metabolite is the liver in which the presence of QCA residues have still been detected after 6 weeks (Rutalj *et al.*, 1996). Unlike its precursors (CBX, desoxy-CBX and monoxy-CBX) QCA has been shown not to be mutagenic, carcinogenic, or cause developmental, embryo, or maternal toxicity *in vivo* (WHO/FAO, 1990; Yoshimura *et al.*, 1981). Through the withdrawal of CBX from Annex B of EU Council Directive 70/524/EEC that governs the use of zootechnical feed additives, the use of CBX is prohibited in the EU from 1st September, 1999. Before the withdrawal of this licence, the MRL for QCA was set at 30 μg kg^{-1} in liver samples. Now that CBX is listed in the banned compounds to be analysed for under the UK National Surveillance Scheme, the presence of any detectable concentration of QCA above the stated Limit Of Quantification

(LoQ) of the assay in a tissue submitted for testing is considered indicative of misuse. Such a positive laboratory test can lead to further sampling of the producer and prosecution if confirmed.

1.14. Olaquindox

1.14.1. Chemistry and physical properties

OQX, 2-(N-2-hydroxyethylcarbamoyl)-3-methyl-quinoxaline-N^1,N^4-dioxide or N-(2-Hydroxyethyl)-3-methyl-2-quinoxalinecarboxamide-1,4-dioxide, has the molecular formula $C_{12}H_{13}N_3O_4$ with a m.w. of 263.25 (see Fig. 1.8.), and has a melting point of 209 °C.

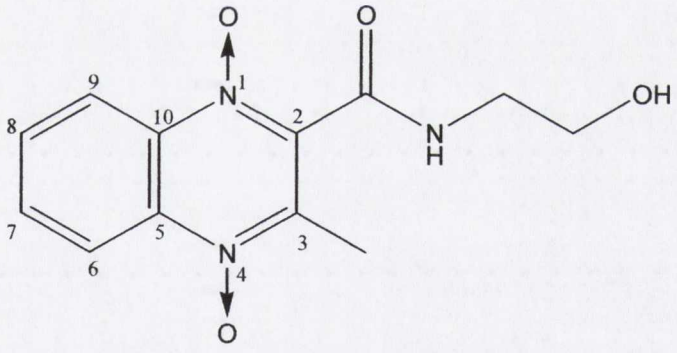

Fig. 1.8. Chemical structure of OQX.

1.14.2. Absorption, metabolism and pharmacokinetics

After a single oral dose (50 mg kg^{-1}), OQX is rapidly absorbed and has been measured in plasma, muscle, liver and kidney of chickens after only 25 minutes (Li *et al.*, 1995). After 2 hours OQX and two mono-*N*-oxides could be identified in plasma (WHO/FAO, 1995). Tissue and plasma concentrations are highest at this time and rapidly decline through

either metabolism or excretion with negligible quantities present after 24 hours (Li *et al.*, 1995). Differences have been found when measuring the levels of OQX and its reduction products in serum and whole blood. This indicates that a portion of OQX and its metabolites binds to corpuscular blood constituents (Fink and Kreisner, 1982), rather than being completely free in the plasma. The majority (~70%) of OQX is excreted unchanged in the urine. Metabolites are also present following limited biotransformation in the liver and kidney (see Fig. 1.9.). The foremost metabolites, resulting from omega oxidation are monoxyolaquindox (monoxy-OQX) and desoxyolaquindox (desoxy-OQX), though several others have been identified (WHO/FAO, 1991; WHO/FAO, 1995). The major detectable residue present in tissue after 24 hours is methyl-3-quinoxaline-2-carboxylic acid (MQCA), with only small or negligible concentrations of the other metabolites present. Residue studies have shown that the depletion of such MQCA from tissues is linear over a 20 day period (WHO/FAO, 1995).

Speirenburg *et al.* (1988a), using pigs given OQX medicated rations (58 mg kg^{-1}), found that concentrations of drug were highest in the stomach with a steady decline along the gastrointestinal tract. Concentrations dropped below the detection limit in the colon suggesting that OQX is continuously absorbed throughout the gastrointestinal tract. Like other quinoxaline-N-dioxides, OQX can be reduced to its corresponding monoxy and desoxy derivatives in the gastrointestinal tract by endogenous enzymes and bacteria (Suter *et al.*, 1978). This opens the possibility that absorption is not complete and that reduction accounts for a portion of the decline in OQX content throughout the gastrointestinal tract, though unlikely, as the majority of OQX is present unchanged at excretion.

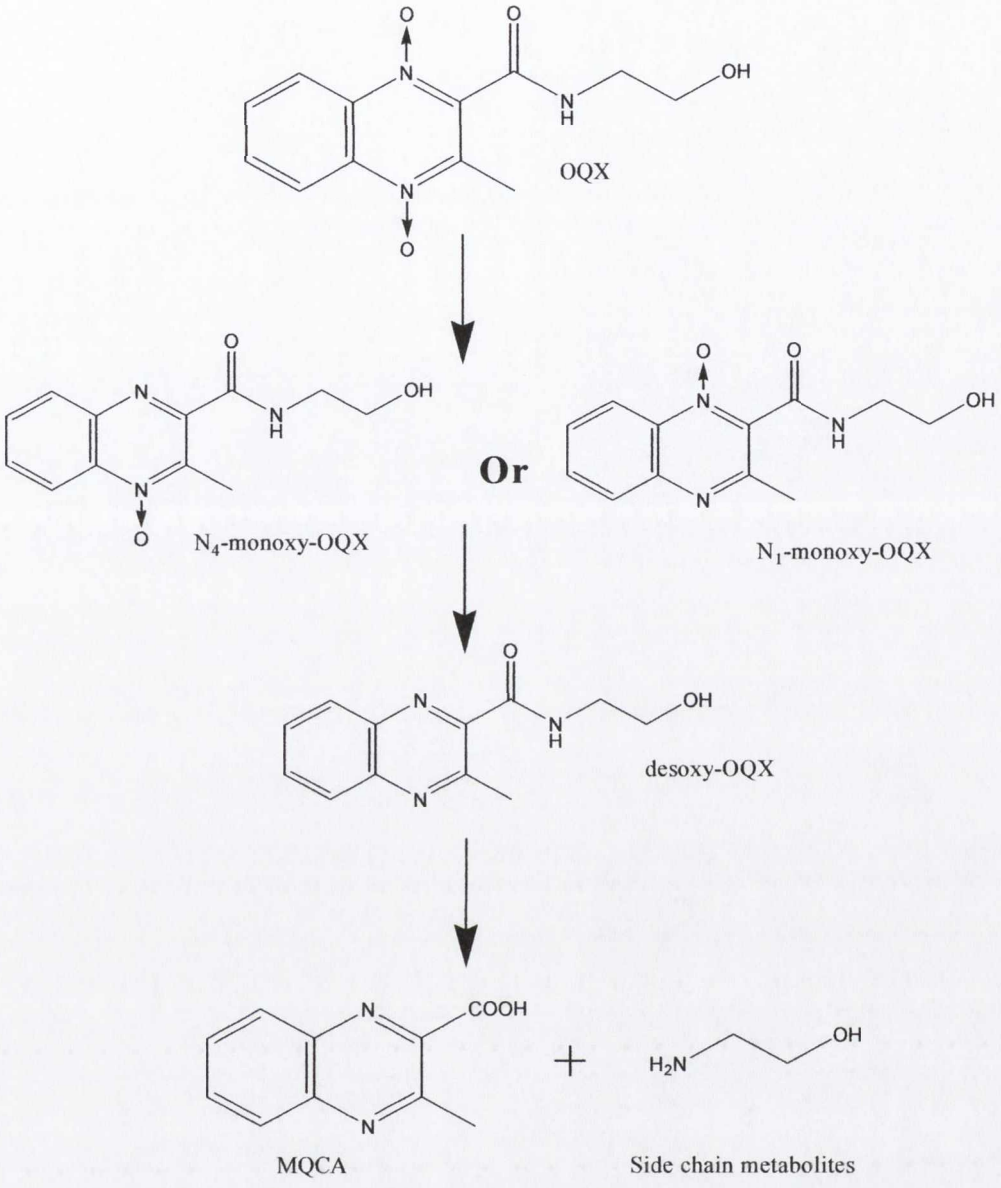

Fig. 1.9. Simplified metabolism pathway of OQX.

1.14.3. Growth promoting action

OQX has been shown to increase significantly the daily weight gains and feed conversion efficiency in young pigs when administered as a feed additive (100 mg kg^{-1}) in comparison to an unmedicated (control) group (Pfirter *et al.*, 1978). One group of 60 piglets was fed OQX at infeed concentrations of 50 mg kg^{-1} over a 28-day period. They

demonstrated a 77.9% increase in weight gain and a 29.3% increased feed utilisation compared to piglets fed identical unmedicated rations over the same time period. In a similar number of piglets fed CBX infeed (50 mg kg^{-1}), little difference was seen in the weight gains and feed utilisation effects in comparison to OQX (Broz *et al.*, 1978). OQX is also reported to affect the release and production of aldosterone in adrenocortical tissue *in vitro* (Jager *et al.*, 1994; Van der Molen *et al.*, 1989b), though the effect of the N-reduced metabolite desoxy-OQX has not been described. OQX, being a quinoxaline-N-dioxide like CBX - with similar growth promoting and aldosterone secretion effects - could be hypothesised to have a mode of growth promoting action similar of CBX, i.e. affecting the secretion of steroid hormones (see Section 1.13.3.). However, whether OQX and/or desoxy-OQX inhibit the same or different enzymes in the neogenesis pathway for aldosterone and cortisol has not been elucidated. Studies reporting increases in weight gain, feed efficiency and nitrogen retention etc. similar to CBX imply that it is likely the same C_{18}-hydroxysteriod deydrogenase and/or 21β-hydroxylase enzymes that are affected.

1.14.4. Veterinary use and clinical symptoms

Like other quinoxaline-N-dioxides, OQX has an antimicrobial action against gram-negative bacteria and also acts as antitreponemal agent, (Williams and Shively, 1978) through bacteriostatic action on the growth of *Treponema hydysenteriae* (Williams and Babcock, 1976) - the cause of swine dysentery. Pharmacokinetics studies in pigs have shown that OQX concentrations are highest in the stomach and decline steadily along the gastrointestinal tract. These concentrations, when compared to the MIC for *Treponema* spp., show that continuous medication at approximately 100 mg kg^{-1} per day would be necessary for a prophylactic effect (Speirenburg *et al.*, 1988a). However, doses slightly

above 100 mg kg^{-1} have been shown to have a high risk of serious side effects to the animal. Consequently, the therapeutic window for OQX use as a prophylactic is small and the risk of accidental overdose is high. In cases of OQX overdose, clinical symptoms reported appear similar to that of CBX. A markedly decreased plasma sodium to potassium ratio, dehydration, reluctance to eat, posterior paralysis, hard faecal matter, damage of the zona glomerulosa in the kidney and increased adrenal capsule volume (Newsholme *et al.*, 1986). These symptoms could be hypothesised to be caused by continuous elevation of aldosterone production and the subsequent secondary effects on the RAS as found with CBX overdose (see Section 1.13.4.). This is supported by studies showing dose-dependent decreases in aldosterone production, hypoatraemia, hypochloraemia, hyperalaemia and hydropic degeneration of the adrenal cortex in piglets fed OQX medicated rations (WHO/FAO, 1991).

1.14.5. Mutagenic properties and antibacterial activity

Though OQX shows mutagenic character, it shows less reactivity than CBX. For example, CBX is mutagenic at 0.001 M in agar with *Salmonella typhimurium*, whereas OQX is mutagenic at 0.02 M under the same conditions (Voogh *et al.*, 1980). Thus, though OQX can induce base-pair substitutions and frame shift mutations, approximately 20 times the amount is required to have the same mutagenic effect *in vitro*. (Beutin *et al.*, 1981; Nunoshiba and Nishioka, 1989).

Mammalian studies with OQX have shown similar mutagenic effects as CBX. OQX has been demonstrated to cause *in vivo* cytogenic damage in the foetal liver of pregnant mice (Cihák and Vontorková, 1985) and induce micronucleated polychromatic erythrocytes in rats (Cihák *et al.*, 1983). The genotoxicity of OQX in the mammalian system has been

demonstrated *in vivo* with chromosomal aberrations and gaps reported in the bone marrow cells of mice (Cihák and Vontorková, 1983). Positive results for OQX using the *rec* and *uvr* assays demonstrate that the bacterial DNA is damaged by UV-induced-like dimers and not just single strand breaks (Yoshimura *et al.*, 1981). Consequently, OQX must directly attack bacterial DNA interfering with the DNA repair process and therefore is genotoxic.

The mutagenicity of the quinoxaline-N,N-dioxides and their ring metabolites has been demonstrated to be dependent on the presence of the N-oxide groups substituted for carbon on the 1st and 4th positions of the quinoxaline ring. Partially reduced quinoxaline-N-oxide exhibits lower mutagenic properties than the N,N-dioxide form and fully reduced derivatives do not show any mutagenic properties (Beutin *et al.*, 1981). Thus, monoxy and desoxy metabolites of OQX are suspected mutagens and thought to possess carcinogenic properties. OQX's genotoxic potential and antibacterial activities are also suspected of being linked to the ability of the drug, through the N^1,N^4-dioxide groups, to cause base-pair substitutions and frameshift mutations in gram-negative bacterial DNA encountered in the gastrointestinal tract. Such conjectures have still to be proved.

1.14.6. Phototoxicity

Drug molecules can induce skin reactions similar to sunburn and edema. These are comparable to allergic contact reactions, with the UVA induced photoproduct of the compound usually being the sensitising agent. This may occur after physical contact with the drug or following medication. Such photoproducts are usually formed in the upper dermal layers of the skin, and can specifically or indiscriminately react with cellular structures and constituents causing an inflammatory reaction. The largest proportion of such damage will occur *in situ* to its formation. However, it may also be found in other

organs and tissues if elevated concentrations are produced in proximity to the bloodstream.

OQX is a derivative of QDN, which was withdrawn from use after reports of photocontact dermatitis in stockmen who were exposed to it during the course of their work. The phototoxicity of this compound is acute and has caused both transient and persistent light reactions lasting over four years in some reported cases (Zaynoun *et al.*, 1976). It is not surprising that OQX has been reported to show similar photoallergic properties. Francalanci *et al.* (1986) reported a possible case of OQX photosensitivity in a pig farm worker employed in the preparation and distribution of OQX medicated feed. Red scaly lesions were present on light exposed parts of the body such as the back and sides of the neck and the dorsa of the hands. A positive photopatch test confirmed that this was a photoreaction to OQX. Further cases of photoallergy after exposure were reported in the 1980s and 1990s. These again showed lichenification of the head, neck and hands upon exposure to sunlight, with the symptoms lasting for considerable lengths of time after the initial onset of the photoallergy (Bedello *et al.*, 1985; Dunkel *et al.*, 1990; Kumar and Freeman, 1996). All safety data sheets for the handling of OQX recommend that contact with OQX or OQX containing products should be avoided, and the photoallergic effects can be attributed to a disregard of such guidelines in some of the cases. A small percentage of the cases have been attributed to sensitisation via airborne particles, through the dust generated by mixing or handling of mealy OQX medicated feeds. This has been reported to induce airborne photoallergic contact dermatitis on exposed parts of the body in "sensitive" individuals (Schauder *et al.*, 1996). The mechanism by which OQX induced photosensitivity was elucidated in the early 1990s. The HPLC analysis of the urine of rats fed OQX (60 mg kg^{-1}) over four days, showed a significant decrease in the observed

amount of OQX excreted between animals exposed to UV light and those kept in the dark (De Vries *et al.*, 1990). A profound change in the metabolism of OQX is thought to occur, with the formation of an oxaziridine derivative through the reaction of OQX with UV light in the dermis. This reaction type is also seen with other photoallergy inducing agents such as chlorpromazine. Such derivatives are highly reactive and can damage proteins, membranes, and other cellular constituents near to their formation leading to such dermal effects being observed. Glutathione (GSSG) is thought to reduce the OQX oxaziridine derivative (as also seen in oxaziridine formation in other imino-N-oxides) to OQX-4-monoxide (see Fig. 1.10.). This is supported by the detection of significant amounts of OQX-4-monoxide in the urine of UV exposed rats (De Vries *et al.*, 1990). In normal OQX metabolism this metabolite is only present in urine in small concentrations, the majority converted to desoxy-OQX and MQCA (De Vries *et al.*, 1990).

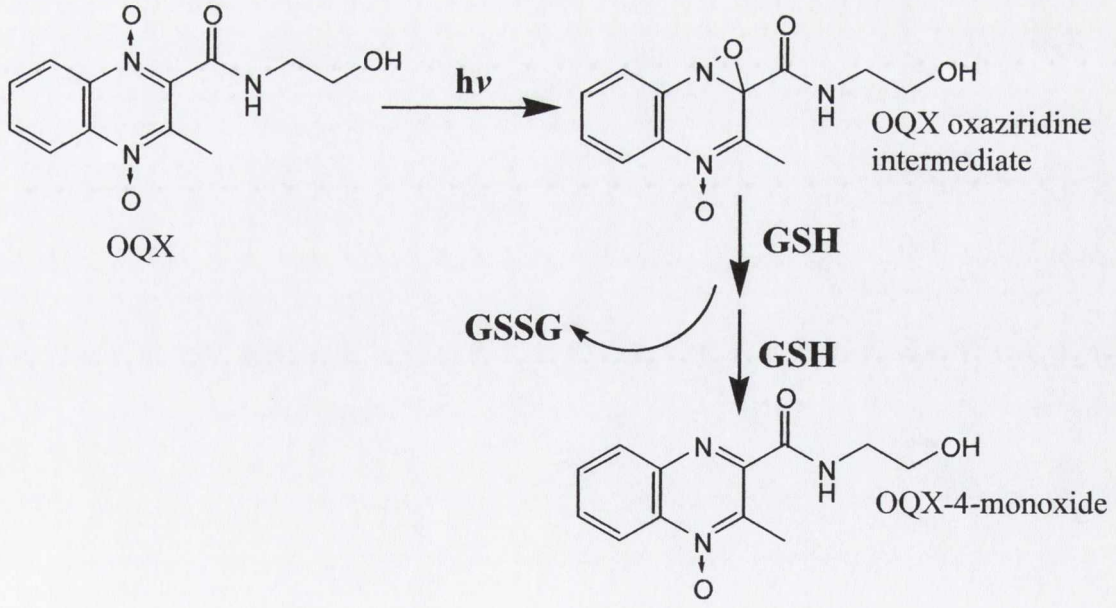

Fig. 1.10. Reaction of OQX with UV light and the formation of OQX-4-monoxide.

1.14.7. EU legislation concerning OQX

OQX was first listed as a zootechnical feed additive under Part F of the Annexes to EU Council Directive 70/524/EEC in 1976 by EU Council Directive 76/933/EEC (European Commission, 1976). EU authorisation for the conditions of its use was also granted in the same year at feed concentrations of 50 mg kg^{-1} (European Commission, 1987b).

In the 1980s and 1990s, several published papers reported both photoallergic and photosensitive effects of OQX in humans (see Section 1.14.6.). This culminated in a report published in November 1992 implicating OQX in cases of photoallergic contact dermatitis in pig producers and farm workers. Other reports published at the same time reported the *in vitro* and *in vivo* genotoxic and germ cell mutagenic properties of OQX and its metabolites (see Section 1.14.5.).

On 14th February, 1997, the Federal Republic of Germany requested a re-evaluation of all quinoxaline-N-dioxides, over concerns of possible risks to the consumer, operators and pigs. After further studies, OQX was classified as a genotoxic agent and possible germ cell-line mutagen, in addition to being a direct danger to both feed factory workers and swine producers through its photoallergic properties. The EU decided to withdraw OQX from use along with CBX on 31st August, 1998 (European Commission, 1998). OQX medicated feed was freely available in the Republic of Ireland up until 1st September, 1998, though not within the UK. It is still legally produced under licence as premixed feed in the USA, Canada and other parts of the world. OQX does not come under EU Council Regulation No 2377/90, as it was never classified as a veterinary medicine but rather a zootechnical feed additive. Therefore, it has been removed from Annex B of EU Council Directive 70/524/EEC, but not added to Annex IV of EU Council Regulation No 2377/90

through separate legislation. The current EU prohibition on OQX use should be enforced by the monitoring for the presence of the marker residue, MQCA, in livestock at slaughter.

1.14.8. Methyl quinoxaline carboxylic acid

MQCA, which has the molecular formula $C_{10}H_9N_2O_2$, has a m.w of 189.193 and is soluble in alcohols and slightly soluble in water. It is a quinoxaline ring with a carboxylic acid group attached to the 2nd carbon of the ring and a methyl group attached to the 3rd carbon of the ring (see Fig 1.11.). Like QCA, it has no N-dioxide groups.

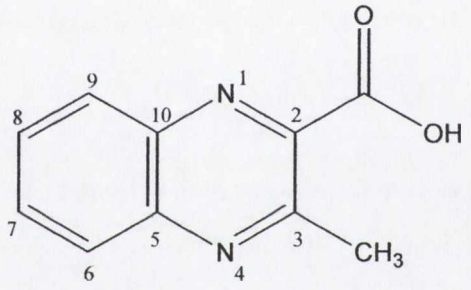

Fig. 1.11. Chemical structure of MQCA.

OQX is extensively metabolised in pigs and the concentration of metabolites has been found to vary notably between different tissue types and animal species. MQCA was found to be the longest remaining tissue metabolite, *in vivo* after OQX medication. As MQCA tissue depletion is linear over time, MQCA has been assigned as the marker residue for OQX use by the FAO and WHO (WHO/FAO, 1995). The mutagenic effects of the quinoxaline-N-dioxides have been attributed to the reduction ability of N-oxide groups present on the ring structure. The toxicity/mutagenicity of MQCA is not yet known, however, MQCA is unlikely to exhibit such properties, as no N-oxide groups are present. The structure of MQCA differs only from that of QCA by a methyl group on the 3rd carbon of the quinoxaline ring. QCA has been shown not to be mutagenic, carcinogenic,

embryo lethal or a developmental antagonist (see Section 1.13.5.). Consequently, it may be suggested that MQCA is also unlikely to exhibit such effects.

The use of OQX was prohibited in the EU as from 1st September, 1999. No MRL or MRPL for MQCA has ever been set; therefore, any detected concentration of MQCA in a sample above the LoQ of the assay is indicative of OQX use. Such a positive result could lead to further sampling of the producer by the regulatory authorities and subsequent prosecution in a court of law. However, OQX is not currently monitored by UK statutory or non-statutory National Surveillance Schemes and implementation of a standard residue test is still to be carried out in many EU countries.

1.15. Analytical methods

1.15.1. Detection of carbadox in feedingstuffs and tissue

In the 1970s detection methods for CBX were largely concerned with the analysis of feed (Goras, 1979; Luchtefeld, 1977; Thorpe, 1976; Thorpe, 1978). This involved the extraction of the drug from the matrix by a solvent followed by a form of column chromatography and then UV/Vis detection. For example, Goras *et al.* (1974) extracted CBX from the matrix with chloroform-methanol solution (3 : 1 v/v), followed by solvent–solvent extraction using potassium phosphate solution (1 M). The sample was applied to an activated alumina column prepared with dimethylformamide (DMF), with CBX forming a yellow band on the column. This was eluted with further volumes of chloroform-methanol solution (3 : 1 v/v). The organic eluate was then back extracted in sodium hydroxide solution (0.1 M). In sodium hydroxide solution, CBX formed a strong chromophore that could be measured against a standard at 420 nm.

Such methods were slow and laborious, but with the development and routine application of HPLC in the 1980s allowing faster analysis times, this increasingly became the separation method of choice. In the 1980s many of these feed methods were based on a simple sample clean-up followed by HPLC with UV detection (Aerts and Werdmuller, 1987; De Graaf and Spierenburg, 1985; Roybal *et al.*, 1985). Lowie *et al.* (1983) used a methanol-acetonitrile solution (50 : 50 v/v) extraction to remove both CBX and pyrantel tartrate from feed. This was followed by alumina column clean up, HPLC and analysis at 365 nm for CBX and 313 nm for pyrantel tartrate. HPLC linked to spectrometric detection could be tailored to allow the detection of one or several other drugs. Several methods have been reported that include CBX (Dos Ramos *et al.*, 1991; McGary, 1986), one method by Thorpe (1980), measured CBX, furazolidone, nitrofurazone and ethopabate all within the same sample.

Fluorescence detection linked to HPLC has been published for CBX and desoxy-CBX in both medicated feed and the contents of the gastrointestinal tract (De Graaf and Spierenburg, 1985). Samples were extracted with a DMF-water solution (95 : 5 v/v) and cleaned up using either gradient elution for the simultaneous assay of both compounds or isocratic elution for the assay of CBX only. Detection of CBX by its native fluorescence (excitation at 310 nm and emission at 487 nm) yielded a sensitive and specific assay allowing greater specificity in the quantification of CBX compared to UV/Vis detection alone.

In the 1980s due to concerns over the mutagenic properties of CBX and related metabolites, development of tissue detection methods became a priority. For example Aerts *et al.* (1988) reported an HPLC method for the pharmacokinetic detection of both

CBX and the monoxy and desoxy metabolites in tissues. A simple liquid-liquid extraction followed by an alumina-florsil cleanup and subsequent iso-octane partitioning was used to prepare the sample. HPLC was used with the post-column addition of an alkali to give a yellow chromophore that can be measured at 420 nm. The method allowed detection at 1.0 $\mu g\ kg^{-1}$ and 5 $\mu g\ kg^{-1}$ for CBX/monoxy-CBX and desoxy-CBX, respectively. This method was further optimised and extended to include other tissue types such as plasma and eggs (Binnendijk *et al.*, 1991). HPLC methods for the detection of CBX in other tissues such as swine muscle and kidney have also been reported (Aerts *et al.*, 1988; Nagata and Saeki, 1991).

Recently HPLC linked to mass spectrometry instead of UV or fluorescence detection has allowed the determination of CBX and 12 other antibacterial reagents (OQX, trimethoprim, clopidol, ormethoprim, morantel, thiamphenicol, pyrimethamine, furazolidone, oxalinic acid, difurazon, nalidixic acid, and piromidic acid) in porcine muscle. After extraction and clean up, a C_{18} column with gradient elution was utilised for the separation and CID was used to induce fragmentation of analyte molecules and enhance the specificity of the method (Fuh *et al.*, 2000).

As previously mentioned (see Section 1.13.2.) CBX, monoxy-CBX and desoxy-CBX, disappear from tissue after $\leq$ 72 hours of the withdrawal of medication (MacIntosh *et al.*, 1985), through either metabolism or excretion via urine or bile. Methods for the direct detection of CBX or related residues would therefore be ineffective in tissue at slaughter, if medication had ceased before this time. This effectively precludes the use of all published detection methods for CBX to pharmacokinetic, pharmaceutical and feedingstuff analysis.

After the withdrawal of CBX as a zootechnical feed additive by the EU on 31st August 1998, all suitable feedingstuff methods need to be capable of analysis at trace concentrations. EU criteria state that methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable on their own as confirmatory methods. This excludes chromatographic separation with UV/Vis detection as a confirmatory method (though HPLC methods may be used for screening feed if they have a sufficiently low detection capability). Therefore in the EU, only the methods based on molecular spectrometry (MS and fluorescence detection) can be used to confirm the presence of CBX in feeds. No currently published methods for the analysis of CBX in animal feedingstuffs meet EU criteria for use as a confirmatory method. A current requirement within the EU is for a confirmatory method for CBX in medicated feedingstuffs.

1.15.2. Detection of quinoxaline carboxylic acid in tissue

One of the earliest methods was published by Lynch and Bartolucci (1982) and involves extraction followed by derivatization and gas-liquid chromatography-MS using SIM. QCA was first isolated from hydrolysates of porcine liver samples by solvent extraction and ion exclusion chromatography. The relative intensities of three ions were monitored: the base peak at *m/z* 130, a second mass at *m/z* 158, and the molecular ion ($M\text{-}H^+$) at *m/z* 188. This was adopted as the reference method in the EU for the determination of QCA in tissue (Commission of the European Communities, 1992). Using a similar extraction method Lauridsen *et al.* (1988) applied the increased detection capability of gas chromatography linked to electron capture mass spectrometry to quantify QCA at lower concentrations (~ 10 $\mu g\ kg^{-1}$). Lynch *et al.* (1988) again using a similar extraction method

further improved on this technique and reported a method using analysis via ion trap detector (TID) connected to a capillary GC using CI conditions. A tetradeutrated internal standard was included in the method to compensate for losses during the extraction procedure. This allowed QCA detection down to 3 μg kg^{-1}. Van Ginkel *et al.* (1990) described a tissue method based on the enzymatic digestion of the tissue, derivatization of the extract and clean up using HPLC followed by GS-MS analysis. Subsequent confirmation of QCA was at *m/z* 102, 130 and 158 with calculation of the in-sample concentration by comparison of the QCA ion at *m/z* 130 to tetradeutrated QCA at *m/z* 134. This method now allowed detection of QCA as low as 1-2 μg kg^{-1}.

Initially the GC-MS methods were used in preference to HPLC as the separation of QCA was recognised as difficult and consequently hard to apply. Rose *et al.* (1995) using the extraction method developed by Van Ginkel *et al.* (1990) and a cation exchange procedure, produced an HPLC method linked to UV/Vis detection. This offered significant advantages over GC-MS methods, as costly equipment was not necessary to apply the technique to surveillance and monitoring. However, an MS method would still be required for confirmation of positive samples.

The EU has published criteria that must be applied to the confirmation of residues in animals and animal products of food origin. None of the previously published methods for detection or confirmation of QCA in tissues meets such stringent requirements. Furthermore they are slow, cumbersome and do not have the advantage of being able to handle large numbers of samples. A current requirement within EU is for a rapid, sensitive QCA confirmatory method that will meet all the criteria for method validation.

1.15.3. Detection of olaquindox in feedingstuffs and tissue

In the late 1970s the analysis of OQX was described by Bories (1979), who recognised that HPLC could be applied to the analysis of OQX in feed to give a fast, accurate and reproducible method of analysis. OQX was first extracted from the feed matrix by homogenisation with potassium carbonate in acetonitrile. OQX's polar character was exploited using an iso-octane partition that removed many common non-polar interfering substances. Reverse phase chromatography was carried out using a Sperisorb (250 x 21mm) 5µm C_{18} LC column and a simple methanol-water mobile phase (5 : 95 v/v) at a rate of 1.2 ml min^{-1}. Before this time OQX feed analysis was mainly carried out using time-consuming TLC that involved the extensive clean up of crude feed extract to eliminate interferents (Fink and Kreisner, 1982).

In the 1980s as HPLC replaced TLC as the separation method of choice. Thente and Anderson (1982) described a feed method that did not involve prior extract purification, however, though rapid, due to the removal of the liquid – liquid partition step, subsequent clogging of the HPLC column limited the usefulness of the method. The Analytical Methods Committee (1985) of the Analyst produced a similar simple single step extraction procedure for the HPLC of OQX in feeds. In the same year Botsoglou *et al.* (1985) reported a simple single step method for OQX analysis in feed using aqueous extraction, filtration, and then analysis by 2nd derivative UV spectrophotometry. However, by this method of analysis estimations of the drug level in the feed sample were only possible.

The above-mentioned methods only allow quantification of the drug at feed medication levels and did not permit analysis down to trace levels. The published methods were also

only concerned with the analysis of OQX in meal. Nagata and Saeki (1987) recognised this problem and reported a method for the extraction, and clean up, in swine tissue. OQX was extracted with acetonitrile and dried down, followed by clean up using alumina column chromatography. HPLC analysis with ultraviolet detection at 350 nm was on Nucleosil C_{18} LC column using a simple acetonitrile/water (5:95 v/v) mobile phase. The detection limit for this method was 0.02 ppm in tissue. This was followed by Speirenburg *et al.* (1988a) using HPLC for the determination of OQX in both medicated feeds and the contents of the porcine gastrointestinal tract. Samples were extracted with water and cleaned on disposable solid phase extraction columns under isocratic conditions. This method allowed investigation of the concentration profiles of OQX in the gastrointestinal tract of pigs fed medicated feed, to evaluate the potency of OQX against *Treponema hyodysenteriae*. The presence (in the gastrointestinal tract) of some N-O reduced metabolites of OQX was also assessed.

Recently, mass spectrometry linked to HPLC has been applied to the analysis of OQX in swine feed and meat samples by Fuh *et al.* (2000). This method allowed the determination of 13 different anti-bacterial compounds in the same sample. CID was used to induce fragmentation of analyte molecules and enhance the specificity of the method and SIM was employed for quantitative determination.

OQX is rapidly metabolised *in vivo* to MQCA, limiting the usefulness of methods for the detection of OQX to pharmaceutical and medicated feedingstuff analysis. After the withdrawal of OQX as a zootechnical feed additive by the EU on 31st August, 1998, all suitable feedingstuff methods need to be capable of analysis at trace concentrations. EU criteria for confirmatory techniques require that specific structural information be

provided by the method of analysis. This precludes all currently published HPLC methods with UV/Vis detection to be used as a means of screening feed for the presence of OQX, if they have a sufficiently low detection capability. Only methods based on molecular spectrometry (mass spectrometry and fluorescence detection) can be used for confirmation of OQX in feeds. No currently published methods for the analyses of OQX in animal feedingstuffs meet all current EU criteria for use as a confirmatory method. A current requirement within the EU is for a confirmatory method of OQX in medicated feedingstuffs.

1.15.4. Detection of methyl quinoxaline carboxylic acid in tissue

Problems with CBX residues and their detection have already been recognised and to an extent addressed, as CBX was in wide-spread use through out the world as a zootechnical feed additive in the 1980s and 90s. However, the same is not true of OQX and MQCA. In addition to this, MQCA was only established as the marker residue for OQX in 1995 and many NRLs have yet to develop and implement confirmatory methods for use in their National Surveillance Schemes. A current requirement within EU is for a rapid, sensitive MQCA confirmatory method that will meet all the criteria for method validation.

1.16. Objectives

Member States must implement monitoring schemes for drug residues in animals and animal products in order to comply with EU regulations. Such legislation also contains lists of compounds to be tested in addition to rules for the use and performance of analytical screening and confirmation methods. If a method does not currently exist or does not meet current regulations, a previously applied analytical method must be improved or a new method developed. Ideally, each Member State NRL would be able to

analyse all the licensed veterinary products to ensure compliance with all EU regulations and enforce any ban on the use of a compound. However, this is not possible in practice and is still a distant prospect for some laboratories. National monitoring programmes often reveal specific problems involving a drug or related residues within their agricultural sector. The confirmatory analytical methods reported in literature for the analysis of such compounds are not always suitable through the publication of new regulations, changes in analytical technology or marker residue. In such cases, steps should be taken by the individual Member State to resolve such problems. The work presented in this thesis addresses several such problems found in NI and the EU.

The objectives of the work were as follows:

1 **(A)** To develop a rapid confirmatory method at contamination levels for the zootechnical feed additives, CBX and OQX in porcine rations as EU Commission Regulation No 2788/98 prohibits the use of either compound in livestock farming following the withdrawal off their product licences.

(B) To validate the developed method as defined by the current regulations for analytical method validation laid out in EU Commission Decision 2002/657/EC, to allow application of the method to the statutory testing of samples in NI taken under the UK National Surveillance Scheme complying with EU Council Directive 96/23/EC.

2 **(A)** To develop a fast and robust confirmatory method for the CBX marker residue in porcine tissue, QCA, due to the prohibition of the use of CBX in livestock farming.

(B) To validate the developed method as defined by regulations for EU analytical method validation as laid out in EU Commission Decision 2002/657/EC.

(C) To compare the developed method to the current in-house GC-MS method and to allow application to the statutory testing of samples in NI taken under the UK National Surveillance Scheme and Meat Inspection Scheme complying with EU Council Directive 96/23/EC.

3 **(A)** To investigate if the CBX metabolite, QCA, can be transferred from a group of medicated animals to a group of unmedicated animals through the use of the same housing, leading to a 'violative' laboratory result, as previously demonstrated with the antimicrobial furazolidone by this laboratory (McCraacken *et al.*, 2000).

(B) If such transfer occurs, to investigate possible ways of differentiating between residues generated due to contamination of the housing and deliberate medication of the animals.

4 **(A)** To develop a fast and robust confirmatory method for the OQX marker residue in porcine tissue, MQCA, due to the prohibition of the use of OQX in livestock farming.

(B) To validate the developed method as defined by regulations for EU analytical method validation as laid out in EU Commission Decision 2002/657/EC, so the method may be applied to the statutory testing of samples in NI taken under the UK National Surveillance Scheme and Meat Inspection Scheme complying with EU Council Directive 96/23/EC.

Chapter 2

Simultaneous determination of carbadox and olaquindox in animal feeding stuffs using liquid chromatography electrospray tandem mass spectrometry

2.1. Introduction

The objectives of the work presented in this chapter were:

(A) To develop a rapid confirmatory method at contamination levels for the zootechnical feed additives, CBX and OQX in porcine rations as EU Commission Regulation No 2788/98 prohibits the use of either compound in livestock farming following the withdrawal off their product licences.

(B) To validate the developed method as defined by the current regulations for analytical method validation laid out in EU Commission Decision 2002/657/EC, to allow application of the method to the statutory testing of samples in NI taken under the UK National Surveillance Scheme complying with EU Council Directive 96/23/EC.

Both CBX and OQX were used throughout the 1980s and 90s in the livestock industry and veterinary medicine as zootechnical feed additives and anti-microbial agents throughout the world. CBX's use as a growth promoter, was in addition to its prophylactic properties in the prevention of swine dysentery and bacterial enteritis in young swine (see Chapter 1.13.). OQX is a quinoxaline 1,4-dioxide structurally related to CBX, but was mainly sold only as a growth stimulant (see Chapter 1.14.). The use of both as feed additives was at medicated concentrations infeed from 50 – 100 mg kg^{-1}. In 1998, both drugs were prohibited from use in livestock production within the EU due to carcinogenic and mutagenic concerns (European Commission, 1998). Any sample taken within the EU generating a result other than zero is therefore considered non-compliant. CBX and OQX are still licensed for use as feed additives in other non-EU countries and are available as premixed zootechnical feed products e.g. Mecadox 10 (Pzifer) and Bayo-N-OX (Bayer). Thus, the potential for illegal use of both drugs still exists inside the EU. Publications have

shown that drugs at low levels can be carried over from one batch of medicated feed into several subsequent batches of unmedicated feed (Kennedy *et al.*, 1998a; Lynas *et al.*, 1998), causing unwanted and illegal residue levels in tissue (Blanchflower *et al.*, 1993b). It is therefore important that the method is capable of determination of both drugs at low levels in feed, to aid both the feed industry and national authorities to spot sources of contamination and eliminate them.

Previously published methods for the detection of CBX or OQX in feed have been based on HPLC coupled to UV or fluorescence as a means of detection. New EU regulations for systemic method validation were published in 2002 superseding all previous criteria (see Section 1.11.). None of the currently published methods met these new criteria for the confirmation of either drug. This chapter describes a method that allows the confirmation of both banned compounds within the same sample using LC linked *via* electrospray to MS-MS and meets new EU criteria. The accuracy and precision were assessed at low concentrations to mimic contamination levels in feed (0.5–5.0 mg kg^{-1}). The simple extraction followed by MS analysis offers considerable advantages in terms of the number of samples that can be processed by an operator in one batch.

2.2. Experimental

2.2.1. Materials

All solvents were of HPLC grade and all other chemicals were of analytical reagent grade. Distilled or de-ionised water was used throughout the study. Both CBX and OQX were obtained from ICN Biochemicals Ltd (Oxfordshire, UK). The mobile phase was acetonitrile-water-formic acid (17.5 : 82.4 : 0.1 v/v), which was filtered and degassed

under vacuum through a 0.45 μm filter and stable for 1 month when stored at room temperature.

2.2.2. Standards

All standards were prepared in amber glassware. Stock standard solutions (1.0 mg ml^{-1}) of both drugs were prepared in N,N-dimethylformamide (DMF) and were stable for at least 6 months. A mixed intermediate standard solution (10 μg ml^{-1}) was prepared by pipetting a volume (1 ml) of each stock standard solution in to a 100 ml volumetric flask, which was then made up to the mark with acetonitrile. Mixed working standard (1.0 μg ml^{-1}) was prepared by dilution of the mixed intermediate standard with acetonitrile. Mixed standards were stable for at least 3 months. Standard solutions were all stored at 4°C in amber vials.

2.2.3. Spiking solution for validation

Spiking solution 1 (50 μg ml^{-1}) was prepared by pipetting a volume (5 ml) of each stock standard solutions (1.0 mg ml^{-1}) into a 100 ml volumetric flask, which was then made up to mark with acetonitrile. Spiking solutions 2 (25 μg ml^{-1}) and 3 (5 μg ml^{-1}) were prepared by dilution of spiking solution 1 (50 μg ml^{-1}) with acetonitrile. Spiking solutions were all stored at 4 °C in amber vials and were stable for at least 3 months.

2.2.4. Equipment

Electrospray LC-MS-MS System

The HPLC system consisting of a Hewlett-Packard (Stockport, Cheshire, UK) series 1100 LC pump, autosampler and solvent degasser were coupled via an electrospray interface to

a Quattro LC (Micromass, Wythenshawe, UK). The LC column used was a Luna 5μ C_{18} 250 x 4.6 mm (Phenomenex, Macclesfield, Cheshire, UK) with a 5μ C_{18} column guard (Phenomenex, Macclesfield, Cheshire, UK) attached. The mobile phase was pumped at a rate of 1 ml min^{-1}, with the column eluant being split so that approximately 200 μl min^{-1} entered the mass spectrometer. The mass spectrometer was run in electrospray positive mode, with the MS source maintained at 150 °C. Nitrogen was used as the drying and nebulising gas at a flow rate of ~600 and 80 l h^{-1}, respectively. The injection volume was 50 μl and the total run time per sample was 15 min. Spectra for CBX and OQX were obtained over the range *m/z* 50-300 with the instrument configured for MS only.

2.2.5. Sample Extraction

Samples of meal were pulverised, if necessary, in a knifetec 1095 sample mill (Tecator, Hoganas, Sweden). Aliquots of test or control meal (10.0 ± 0.1 g) for analysis were weighed into 125 ml polythene bottles. Four recovery samples fortified at 1.0 mg kg^{-1} were prepared by the addition of mixed intermediate standard solution (1ml) to known negative meal. In addition, two known negative samples, two matrix standard samples and two check samples were analysed with every batch. The check samples are samples fortified with CBX and OQX at a concentration unknown to the analyst. Fortified samples for validation purposes were also prepared at this stage by addition of spiking solutions 1, 2 or 3 (50, 25 or 5 μg ml^{-1}, 1 ml) onto known negative meal giving CBX and OQX recovery levels at 5, 2.5 and 0.5 mg kg^{-1}, respectively. The samples were allowed to stand for 10 min before proceeding. Acetonitrile-chloroform solution (50 : 50 v/v, 50 ml) was added and each sample placed on a mechanical shaker for 1 hour. The solutions were centrifuged (2000*g*, 4 °C, 10 min.) and 1 ml of the solvent extract transferred into a clean

3 ml glass tube. Matrix working standards were prepared at this stage by pipetting mixed working standard solution (1.0 μg ml^{-1}, 1 ml) plus matrix standard extract (1 ml) into a clean 3 ml glass tube. The solvent was removed from samples and matrix standards by drying under a stream of nitrogen at 65 ± 5 °C. The remaining residue in each tube was re-dissolved in mobile phase (1 ml) by sonication for 10 min. Aliquots of each solution (200 μl) were transferred into microvials for analysis.

2.2.6. LC-MS-MS analysis

For each sample or matrix standard ran, the instrument was initially configured for multiple reaction monitoring (MRM) of OQX from 0 to 6 minutes. Quadrupole 1 was set to transmit the molecular ion ($[M+H]^+$) of OQX (*m/z* 264) and quadrupole 2 set to transmit the MS-MS product ions at *m/z* 212 and 143. The collision energies were 20 and 35 eV, respectively. From 6 to 15 min the instrument settings were switched to enable for the MRM of CBX. Quadrupole 1 was set to transmit the molecular ion ($[M+H]^+$) of CBX (*m/z* 263) and quadrupole 2 set to transmit the MS-MS product ions at *m/z* 231 and 90. The collision energies were 15 and 30 eV, respectively. The collision entrance and exit energies were set to 0 and 2 eV, respectively for all MRM. The collision gas, argon, was bled into the cell at a pressure of 2.3×10^{-3} mbar. The dwell time for all ions was 0.5 sec. Results for OQX were calculated by comparing the response of *m/z* 264 → 143 (OQX base peak) in the samples with the mean of the *m/z* 264 → 143 response for the matrix working standards (1.0 μg ml^{-1}) in the run which were equivalent to 5.0 mg kg^{-1}. Results for CBX were calculated in a similar way, by comparing the response of *m/z* 263 → 231 (CBX base peak) in the samples with the mean of the *m/z* 263 → 231 response for the

matrix working standards (1.0 μg ml^{-1}) in the run which were again equivalent to 5.0 mg kg^{-1}.

2.3. Results and Discussion

2.3.1. Tandem MS analysis of carbadox and olaquindox

Both CBX and OQX gave a strong response in electrospray positive mode, with prominent peaks for the $[M+H]^+$ ions at *m/z* 263 and 264, respectively; MS-MS of the *m/z* 263 $[M+H]^+$ molecular ion of CBX produced two prominent product ions at *m/z* 231 (base peak) and 90 (Figure 2.1. A). The transition *m/z* 263 → 231 probably results from the loss of the two co-ordinate covalent oxygen atoms from the $[M+H]^+$ molecular ion. The *m/z* 263 → 90 transition may result from the loss of the quinoxaline ring (Figure 2.1.A). The MS-MS of the *m/z* 264 $[M+H]^+$ molecular ion of OQX (Figure 2.1. B) also produces two prominent product ions at *m/z* 212 and 143 (base peak). The m/z 264 → 212 product probably represents the loss of one of the co-ordinate covalent oxygen atoms as well as the loss of H_2O and a hydroxyl. The *m/z* 264 → 143 product ion is thought to be produced as a result of the subsequent loss of the side chain (Figure 2.1. B).

According to the EU's technical criteria for the identification of veterinary drugs a minimum of 4 identification points (IP) are required for the confirmation and unambiguous identification of unauthorised compounds. According to the revised criteria 1.5 IP is given for each MS-MS product ion measured (and 1 IP for the precursor ion). The described method, which measures 2 product ions (+1 precursor ion) for each compound scores 4.0 IPs for CBX and OQX. This fulfils the minimum identification criteria needed.

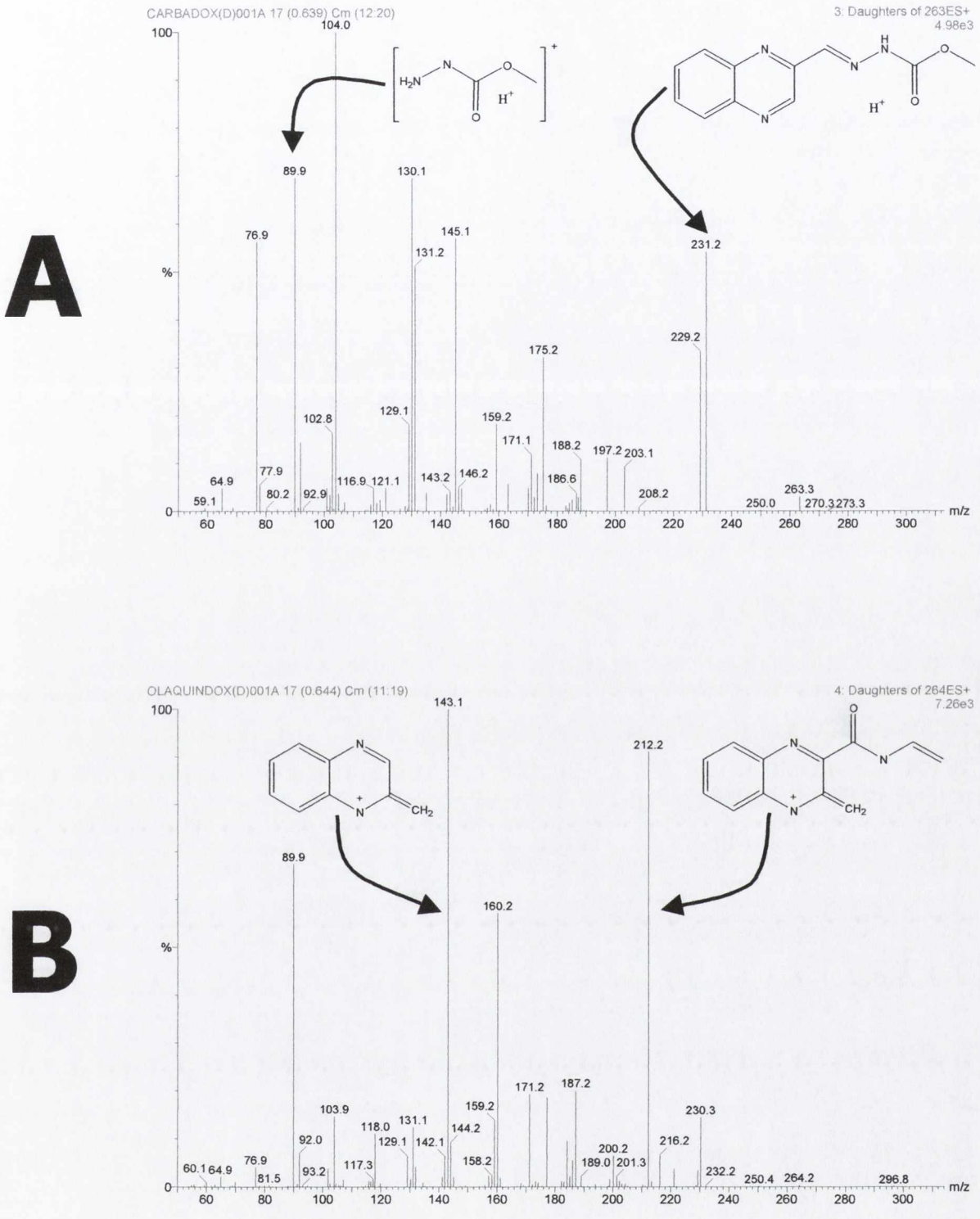

Fig. 2.1. MS-MS spectra of CBX (A) and OQX (B), including the structure of the proposed diagnostic ions.

However, the ion ratios for each unknown sample must also correspond (within predefined limits) to those measured in the standards within the run for unambiguous identification. In this method the ratio of *m/z* 263 : 90 and *m/z* 263 : 231 were measured for CBX with *m/z* 264 : 212 and *m/z* 264 : 143 measured for OQX. All of these results had to meet the pre-set tolerances stated in criteria before they could be considered acceptable (see Chapter 1.11.1). All the samples used in the validation study were subject to, and met, the above stated identification criteria.

Calculation of results is by comparison to standards made with sample matrix, so that any enhancement or suppression of the signal may be compensated for within the run. Such effects can otherwise make the computation of recoveries and results difficult due to variation in the required ion ratios for identification, as well as the absolute peak areas being affected.

2.3.2. Method performance characteristics

This extraction method was developed by simplifying and modifying several published techniques for the extraction of CBX or OQX from feed and combining them to allow the dual extraction of both drugs (Botsoglou *et* al., 1985; Goras *et* al., 1974; Lowie *et al.*, 1983; Nagata and Saeki, 1987). Due to the simplicity of the method and the single extraction step, up to 20 sample could be prepared (including check samples and standards) for analysis within half a working day.

Figure 2.2. shows MRM chromatograms for CBX and OQX standards (equivalent to 5.0 mg kg^{-1}), a negative meal and a meal fortified with CBX and OQX at 0.5 mg kg^{-1}. Both compounds show good chromatography. The negative meal is free of interfering peaks at

the retention times of the analytes and only shows baseline signal over the time window, proving they are truly negative. Standards were prepared by fortifying extracted negative sample matrix, as described above. This approach was used to ensure that any enhancement or suppression of the signal caused by the presence of matrix is compensated for in the standards included in each batch.

Validation of the method was performed by the analysis of different negative porcine feed samples which had been fortified at 3 different levels (n=6 at each level) on each of three days by one operator. Since Commission Decision 2002/657/EC applies to testing for residues in animals and animal products, MRPLs have not been (and will not be) established for CBX and OQX in feed. Consequently, it was not necessary to calculate some of performance characteristics, such as CCα and CCβ, listed in that decision (see Chapter 1.11.5 and 1.11.6). Porcine feeds were used for the validation studies, as this laboratory is required to test only porcine feed for the presence of these compounds. The concentrations of CBX and OQX chosen to assess the accuracy and precision of the method were 0.5, 2.5, and 5.0 mg kg^{-1}. The results for CBX and OQX are presented in Table 2.1. and Table 2.2., respectively. The mean recovery of the assay for CBX was 86.4 % and for OQX was 78.3 %. The Limit of Determination for this method is 0.5 mg kg^{-1} as this is the lowest concentration at which the described method has been validated with a specified degree of accuracy and precision. The Detection Limit (based on a signal: noise ratio of 3 : 1) achievable by this method is approximately one order of magnitude lower for each compound (i.e. approximately 50 μg kg^{-1}). The Detection Limit of this electrospray LC-MS method is comparable to that achieved by Fuh *et al.*, (2000), which claimed a Detection Limit of 0.2 mg kg^{-1} for OQX and 0.05 mg kg^{-1} for CBX.

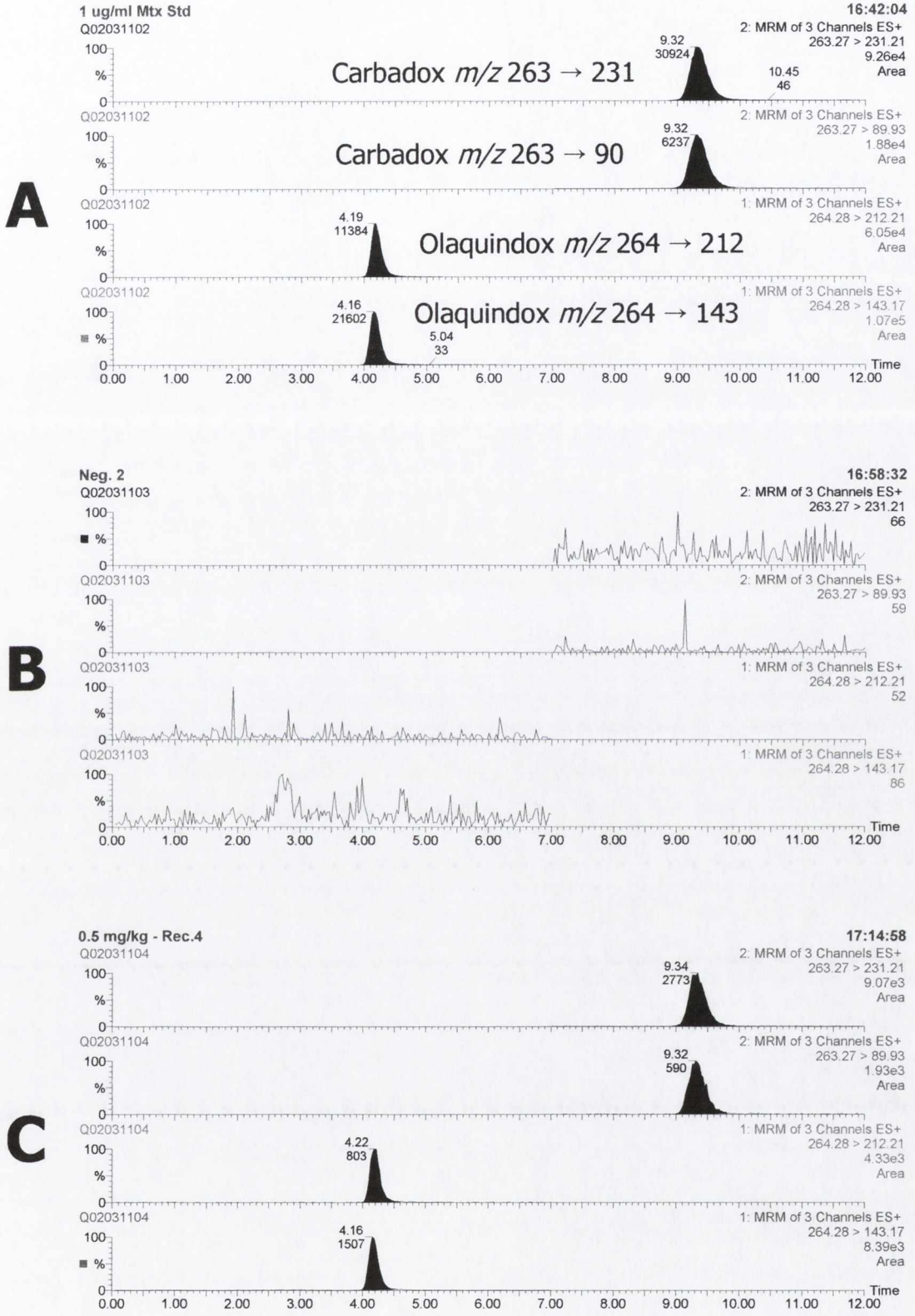

Fig. 2.2. MRM chromatograms for: *A*) matrix standard (equivalent 5 mg kg^{-1}), *B*) a known negative meal and *C*) a negative meal spiked with CBX and OQX at 0.5 mg kg^{-1} for each drug.

Table 2.1. **Inter- and intra-assay reproducibility and recovery for known negative meal spiked with CBX at 0.50, 2.50 and 5.00 mg kg^{-1}.**

	0.50 mg kg^{-1}	2.50 mg kg^{-1}	5.00 mg kg^{-1}
Day 1			
Mean	0.50	2.27	3.97
s	0.04	0.08	0.32
RSD	7.70	3.70	8.20
% Recovery	99	91	79
n	6	6	6
Day 2			
Mean	0.41	1.94	3.51
s	0.02	0.06	0.13
RSD	4.20	3.00	3.60
% Recovery	82	78	70
n	5	6	6
Day 3			
Mean	0.40	1.88	3.53
s	0.02	0.06	0.09
RSD	4.50	3.30	2.60
% Recovery	80	75	71
n	6	6	6
Overall			
Mean	0.44	2.03	3.67
s	0.05	0.19	0.29
RSD	11.74	9.22	7.92
% Recovery	88	81	73
n	17	18	18

Table 2.2. **Inter- and intra-assay reproducibility and recovery for known negative meal spiked with OQX at 0.50, 2.50 and 5.00 mg kg^{-1}.**

	0.50 mg kg^{-1}	2.50 mg kg^{-1}	5.00 mg kg^{-1}
Day 1			
Mean	0.38	2.11	4.06
s	0.04	0.09	0.41
RSD	11.00	4.30	10.00
% Recovery	76	84	81
n	6	6	6
Day 2			
Mean	0.38	1.92	3.65
s	0.04	0.17	0.38
RSD	11.3	8.90	10.30
% Recovery	75	77	73
n	5	6	6
Day 3			
Mean	0.40	1.98	3.85
s	0.02	0.14	0.26
RSD	4.60	7.20	6.70
% Recovery	80	79	77
n	6	6	6
Overall			
Mean	0.39	2.00	3.86
s	0.03	0.15	0.37
RSD	8.78	7.71	9.67
% Recovery	77	80	77
n	17	18	18

Their method was developed to measure these compounds in pork meat. However, the appropriate marker residues for OQX and CBX in meat are not the parent drugs, but are methyl quinoxaline-2-carboxylic acid and quinoxaline-2- carboxylic acid, respectively. Furthermore, the chromatograms presented for the determination of OQX showed the presence of a substantial interfering peak. Finally, the method Fuh *et al.* (2000) did not address the issue of analyte identification by measurement of characteristic fragment ions. Thus, it is not suitable for use as a confirmatory method under current EU legislation.

2.4. Conclusion

Previous publications have shown that low levels of drug can be carried over from one batch of medicated feed into several ensuing batches of unmedicated (blank) feed (Lynas *et al.*, 1998). This contamination of feed with unlisted drugs has been shown to cause 'violative' residue concentrations in tissue, leading to the possible condemnation of all related carcasses and prosecution of an 'innocent' producer.

The method presented is both rapid and simple and will allow measurement of contamination levels of CBX and OQX in both commercially produced meal and experimental formulations. Approximately 20 samples, excluding controls and negatives, can be extracted and prepared for analysis in half a working day. The method has been validated according to EU Commission Decision 2002/657/EC over the concentration range 0.5 – 5.0 mg kg^{-1} for both compounds.

The Limit of Determination for this method is 0.5 mg kg^{-1} as this is the lowest concentration at which the described method has been validated with a specified degree of accuracy and precision. The Detection Limit (based on a signal: noise ratio of 3 : 1)

achievable by this method is approximately one order of magnitude lower for each compound (i.e. approximately 50 $\mu g\ kg^{-1}$).

The method has been applied to the analysis of samples taken in NI under the UK National Surveillance Scheme. The objectives set for this chapter have therefore been fulfilled.

2.5. Publications

The method described in this chapter was submitted to the *Food Additives and Contaminants* in 2003 under the title:

Hutchinson, M. J., Young, P. B., and Kennedy, D. G., Development and validation of a method for confirmation of carbadox and olaquindox in porcine feedingstuffs using LC – electrospray MS-MS.

The method described in this chapter was presented in poster format at the *Fourth International Symposium on Hormone and Veterinary Drug Analysis, Antwerp, Belgium* in 2002 under the same title.

Chapter 3

Determination of the carbadox metabolite, quinoxaline-2-carboxylic acid, in porcine liver using liquid chromatography electrospray tandem mass spectrometry

3.1. Introduction

The objectives of the work presented in this chapter were:

(A) To develop a fast and robust confirmatory method for the CBX marker residue in porcine tissue, QCA, due to the prohibition of the use of CBX in livestock farming.

(B) To validate the developed method as defined by regulations for EU analytical method validation as laid out in EU Commission Decision 2002/657/EC.

(C) To compare the developed method to the current in-house GC-MS method and to allow application to the statutory testing of samples in NI taken under the UK National Surveillance Scheme and Meat Inspection Scheme complying with EU Council Directive 96/23/EC.

National Surveillance Schemes for veterinary drug residues are required to monitor for the use of CBX in animals for human consumption. QCA is the most persistent metabolite of CBX and has been designated the marker residue for CBX use in animals (Ferrando *et al.*, 1975). Before the withdrawal of the product licence for CBX, the MRL for QCA in liver was set at 30 $\mu g\ kg^{-1}$. Now the presence of any confirmed concentrations of QCA in a sample is considered a non-compliant result. No MRPL has yet been set for QCA. The EU has revised the criteria applied to the screening and confirmation of veterinary drug residues in animals of food origin (see Chapter 1.11.). None of the previously published methods meets these new technical criteria. Furthermore, the existing methods are slow, cumbersome and are not applicable to large numbers of samples. The described assay for the confirmation of the CBX metabolite in porcine liver uses liquid chromatography linked *via* an electrospray interface to tandem mass spectrometry and meets all the EU's new technical criteria. The method also offers considerable advantages in terms of the

number of samples that can be processed by an operator in one batch. The accuracy and precision of the method were accessed over a wide concentration range (3-300 μg kg^{-1}) to reflect field samples collected in NI. Correlation between the described method and the in-house routine method, based on high resolution gas chromatography MS, was assessed using a range of naturally incurred samples collected through the residue control schemes in NI.

3.2. Experimental

3.2.1. Materials

All solvents were of HPLC grade and all other chemicals were of analytical grade. Distilled or de-ionised water was used throughout the study. Deuterium labelled Quinoxaline-2-Carboxylic Acid (d_4-QCA) from RIVM (European Union Community Reference Laboratory, Bilthoven, The Netherlands) was employed as an internal standard. QCA was obtained from Sigma-Aldritch Co. Ltd. (Gillingham, Dorset, UK). Stock standard solutions of QCA (1.0 mg ml^{-1}) and d_4-QCA (10 μg ml^{-1}) were prepared in methanol. Working standards for both (1.0 μg ml^{-1}) were prepared by serial dilutions of the stock standards in methanol. Stock standards were stable for 1 year and working standards were stable for 3 months, when stored in amber vials at 4 °C. The back extraction solution was 0.1 M sodium phosphate buffer, pH 8.0. The mobile phase, methanol-water-acetic acid (40 : 59.6 : 0.4 v/v), was filtered and degassed before use.

3.2.2. Equipment

Solid Phase Extraction System

Solid phase extraction (SPE) was carried out on an ASPEC XL4 coupled to a 404-syringe pump (Gilson Ltd, Middleton, Wisconsin, USA) using IST non-endcapped benzenesulfonic acid (SCX), 3 ml, 1.0 g sorbent material, disposable extraction columns (IST Ltd, Mid. Glamorgan, UK). These were wetted with methanol (5 ml, 2.5 ml min^{-1}; 0.3 ml air push, 3 ml min^{-1}) and conditioned with hydrochloric acid (0.1 mol l^{-1}, 5 ml, 2.5 ml min^{-1}; 0.3 ml air push, 3 ml min^{-1}). The sample was applied onto the cartridge (2ml, 1 ml min^{-1}; 3 ml air push, 1 ml min^{-1}) and washed with a further aliquot of hydrochloric acid (0.1 mol l^{-1}, 5 ml, 2.5 ml min^{-1}; 0.3 ml air push, 3 ml min^{-1}). The cartridge was eluted with 0.1 mol l^{-1} sodium hydroxide-methanol solution (70 : 30 v/v) into 10 ml glass tubes (3 ml, 1 ml min^{-1}; 3 ml air push, 1 ml min^{-1}).

Electrospray LC-MS-MS System

All tandem MS analyses were performed using a Quattro LC mass spectrometer (Micromass, Wythenshawe, UK) in positive mode. A Hewlett Packard HPLC system (Stockport, Cheshire, UK) comprising a 1100 series binary pump, autosampler and solvent degasser was coupled *via* an electrospray interface to the Quattro LC. The HPLC column used was a Columbus 5µ C_{18} 150 x 4.60 mm (Phenomenex, Macclefield, Cheshire, UK). The mobile phase was pumped at a rate of 1.0 ml min^{-1} with the column effluent split so that approximately 200 µl min^{-1} entered the mass spectrometer. The total run time was 15 min. The MS source was maintained at 150 °C with nitrogen used as the drying and nebulising gas at a flow rate of 500 and 80 l h^{-1}, respectively. The collision gas, argon was bled into the cell at a pressure of 2.3 x 10^{-3} mbar.

High Resolution GC-MS System

All high-resolution GC-MS analyses were performed using a three-sector double focusing mass spectrometer (VG autospec, VG Analytical, Manchester, UK). A HP5890 gas chromatograph fitted with a HP7673 autosampler (Stockport, Cheshire, UK) was coupled *via* a transfer line to the mass spectrometer. The GC column was a 25m x 0.25mm x 0.25μm Rtx-1 (Restek, Bellefonte, Arizona, USA) capillary column. The injection port temperature was set at 280 °C. The pre-run oven temperature was 100 °C, which was ramped to 200 °C at 10 °C min^{-1}, and then to 280 °C at 50 °C min^{-1}. The run time per sample was 12 min. The instrument was operated in EI positive mode with an electron energy of 70 eV and a trap current of 300 μA. The temperature of the source and transfer line were held at 200 °C and 290 °C, respectively. The magnet was set at a lock mass of *m/z* 198.988 and the instrument calibrated using perfluoronbutylamine.

3.2.3. Tissue Extraction

Aliquots of homogenised test or control liver (5.00 ± 0.05 g) were weighed into 35 ml glass tubes. Two known negative samples were analysed in every batch. In addition, four recovery samples, fortified at 30 μg kg^{-1} were prepared by adding QCA working standard solution (150 μl) to known negative tissue. A further two check samples were analysed with every batch. These were known negative samples that had been fortified with QCA at a concentration unknown to the analyst. Fortified samples for validation purposes were also prepared at this stage by addition of the relevant amount of standard solution. Internal standard, d_4-QCA (150 μl of the 1.0 μg ml^{-1} working standard) was added to each sample. Sodium hydroxide solution (3 M, 10 ml) was added to each sample and all were placed in a water bath set at 100 °C for 30 min. The samples were removed and allowed to cool to

room temperature. Concentrated hydrochloric acid (4 ml) was added to each tube and the contents mixed for 30 sec. Ethyl acetate (6 ml) was added and the tubes invert for 1 min and centrifuged (2000*g*, 4 °C, 10 min.) The upper layer was transferred into a polyethylene tube (50 ml) and the extraction repeated. The two extracts were then combined. Back extraction solution (8 ml) was added and the tubes inverted for 1 min and centrifuged (2000*g*, 4 °C, 10 min.). The upper organic layer was aspirated to waste and an aliquot (4 ml) of the aqueous phase transferred to a 10 ml glass tube containing concentrated hydrochloric acid (1 ml). Solid phase extract was then carried out as described in Section 3.2.2. To each SPE cartridge eluate, concentrated hydrochloric acid (300 µl) was added. Ethyl acetate (2 ml) was added and the tubes vortexed for 15 s and then centrifuged (2000*g*, 4 °C, 10 min.). The upper organic layer was transferred into glass centrifuge tubes. The extraction was repeated a further two times and the extracts were combined. The ethyl acetate extracts were taken to dryness under a stream of nitrogen at 60 °C. Methanol-water solution (10 : 90 v/v, 100 µl) was added, and the tubes were vortexed for 15 sec. The solutions were then transferred to microvials for analysis.

3.2.4. Tandem MS analysis

Spectra for QCA and d_4-QCA were obtained over the range *m/z* 50-200 with the instrument configured in MS mode only. For multiple reaction monitoring (MRM), quadrupole 1 was set to transmit the molecular ion ($[M + H]^+$) of QCA (*m/z* 175) and d_4-QCA (*m/z* 179). Quadrupole 2 was set to transmit the QCA product ions at *m/z* 129, 102 and 75 along with the d_4-QCA product ion at *m/z* 106. The collision cell entrance and exit energies were set at 0 and 2 eV, respectively. The collision energy was optimised at 15, 30 and 45 eV for the QCA product ions at *m/z* 129, 102 and 75, respectively. The collision energy was optimised at 30 eV for the d_4-QCA product ion at *m/z* 106. The dwell time for

each ion was 0.3 sec. Pumping mobile phase for 15-20 min. before analysis equilibrated the system. The injection volume was 30 µl. Working standard standards (QCA, d_4-QCA, 30 µg kg^{-1}) were run on the LC-MS-MS system above until reproducible peak areas were obtained. Aliquots of sample extract were then injected, with a standard after every 4 samples. Concentrations for QCA were calculated by comparing the ratio of the *m/z* 175 → *m/z* 129 response (QCA) with the *m/z* 179 → 106 response (d_4-QCA) in the samples with those in the standards (30 µg kg^{-1}) included in the run.

3.2.5. GC-MS Analysis

For QCA analysis, the instrument was run in single ion monitoring (SIM) mode. The ions at *m/z* 102.034, 130.053, 158.048 and 188.058 were monitored for QCA and *m/z* 192.083 for d_4-QCA. The resolution was set at 5,000 and multiplier voltage at 400 V. The injection volume was 1.5 µl in splitless injection mode. Working standard standards (QCA, d_4-QCA, 30 µg kg^{-1}) were run on the GC-MS system above until reproducible peak areas were obtained. Aliquots of sample extract were then injected with a standard after every 4 samples. Concentrations were calculated by comparing the peak ratio of the base peak (*m/z* 188.058) with the equivalent ion (*m/z* 192.083) for the internal standard in the sample with the mean ratio (*m/z* 188.058 : 192.083) of the standards (30 µg kg^{-1}) in the run.

3.3. Results and Discussion

3.3.1. Tandem MS analysis of quinoxaline carboxylic acid

MS-MS of the *m/z* 175 $[M + H]^+$ molecular ion of QCA (see Fig. 3.1) produces three prominent products at *m/z* 129, 102 and 75. The abundance of each product ion was optimised at collision energies of 15, 30 and 45 eV, respectively. The product ion formed

at *m/z* 129 results from the successive loss of water and carbon monoxide. This is supported by the observation of a small peak at *m/z* 157 (loss of H_2O) in the trace at 15 eV. The other product ions (*m/z* 102 and 75) were formed by two successive losses of HCN from the molecule. New EU technical criteria require a minimum of 4 IPs to unambiguously confirm unauthorised substances in food of animal origin. The criteria score 1.5 IPs for each MS-MS product ion measured, plus 1 IP for the precursor ion (whether measured or not). Thus, the described method, which measures 3 product ions scores 4.5 IPs, plus 1 IP for the precursor ion at *m/z* 175, yields a total of 5.5 IPs.

This more than fulfils the minimum identification criteria required for the method. However, for unambiguous identification, not only do the required number of ions have to be present, but all measured ion ratios in unknown samples must correspond to those in standards within predefined limits (see Chapter 1.11.1.). The maximum permitted tolerance in the ion ratio varies with the relative intensity of the product ions to the base peak. In LC-MS-MS, the maximum permitted tolerances are summarised in Table 1.1. In the method 2 ion ratios were measured for the purposes of analyte identification (*m/z* 102 : 129 and 75 : 129), all of which had to meet these pre-set tolerances before they were considered acceptable. All of the samples used in the validation study met the relevant identification criteria.

3.3.2. Method performance characteristics

The described method is much easier to perform than the standard method described by (Lynch and Bartolucci, 1982). In our experience, a skilled analyst could process 4 samples in duplicate using the older method in 2.5 days (excluding negatives, recoveries and check samples).

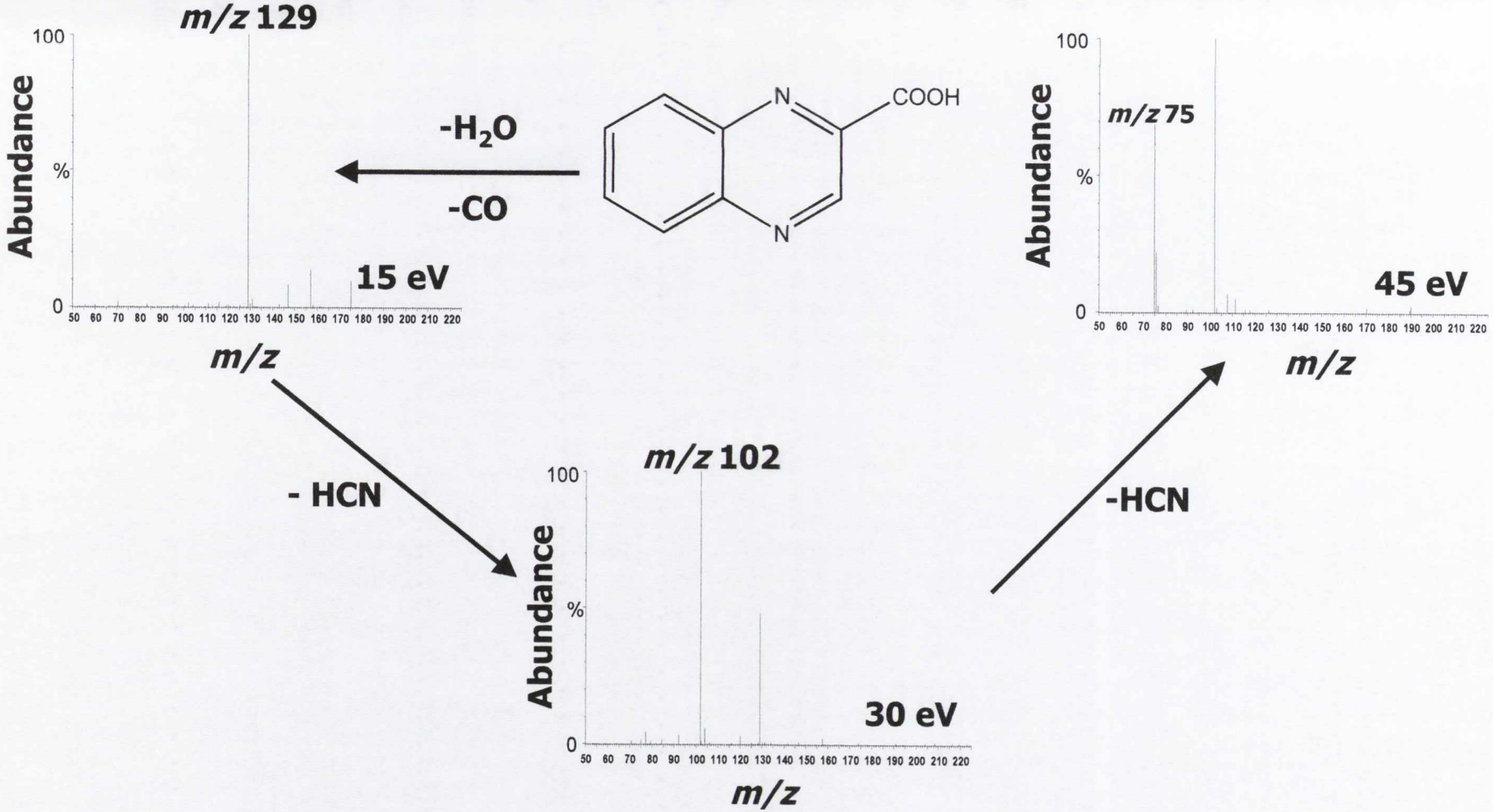

Fig. 3.1. MS-MS spectrogram of the molecular ion $[M + H]^+$ of QCA at *m/z* 175.

The extraction method was developed by improving, updating and modifying several published techniques (Lauridsen *et al.*, 1988; *Lynch* and Bartolucci, 1982; Rose *et al.*, 1995, Van Ginkel *et al.*, 1990). This simplified the method speeding up the extraction process, and also allowed the inclusion of automated solid phase extraction which increased the number of samples that could be analysed per run. The described method has the major advantage of being capable of handling 16 samples in duplicate in 1.5 days (again, excluding control samples). Fig 3.2. shows MRM chromatograms for a QCA standard (30 μg kg^{-1}), a negative liver and a negative liver fortified with QCA at 3.0 μg kg^{-1}at *m/z* 129, 102, 75 and 106 (internal standard, d_4-QCA). The chromatograms show no spurious peaks due to the matrix. The negative meal is free of interfering peaks at the retention times of the analytes and only shows baseline signal over the time window, proving they are truly negative.

The absolute recovery (based on the analysis of 4 negative liver samples fortified with QCA at 30 μg kg^{-1} and carried through the method in the absence of internal standard) achieved by the described method (57.6 ± 5.1%) is comparable with the figure achieved by the GC-MS method (63.2 ± 4.8%). The accuracy and precision of the method was determined over a wide concentration range (3.0 – 300 μg kg^{-1}) on three separate occasions to reflect the QCA concentrations that we have encountered in our statutory control schemes. Over this concentration range, a linear relationship between signal (ratio of transition *m/z* 175 → 129 to transition *m/z* 179 → 106) and QCA concentration (r^2 = 0.9994) was observed. All results were calculated after the application of the identification criteria described in Chapter 1.11.1. These results are summarised in Table 3.1.

The method adopted in this laboratory for the calculation of the limits of detection, CCα and CCβ is discussed separately in Chapter 1.11.5. and Chapter 1.11.6., respectively. For unauthorised compounds, the analytical limits may be determined graphically following the replicate analysis (n = 6) of blank samples fortified at 1.0, 1.5 and 2.0 times the MRPL. The MRPL is a limit established by the EC and CRLs. It is designed to harmonise the analytical performance of methods for authorised substances across Member States. Although an MRPL for QCA has not yet been set, it is likely that it will be one fifth of the former MRL for QCA (30 μg kg^{-1}). We have chosen to assess CCα and CCβ using a lower MRPL (2.0 μg kg^{-1}) to ensure that our QCA method will meet the level finally adopted within the EU. Therefore, 6 known negative liver samples were fortified with QCA at 2.0, 3.0 and 4.0 μg kg^{-1}. The samples were analysed using the described method and the relevant identification criteria were applied.

A graph of signal (*m/z* 129 : 106) *versus* added QCA concentration was constructed (Fig 3.4). CCα was calculated as the concentration corresponding to the signal at the Y intercept plus 2.33 times the standard error of the Y intercept (α = 1%). A further 1.64 times the standard errors of the Y intercept was added to the signal at the Y intercept to calculate the CCβ.

In this way, the CCα, the Decision Limit was found to be 0.16 μg kg^{-1}. This is the lowest concentration that can be distinguished from zero, with an error probability of 0.01. CCβ, the detection capability, being the point at which 95% of samples will be declared violative with a β-error probability of 0.05 and a very low α-error probability, was found to be 0.27 μg kg^{-1}.

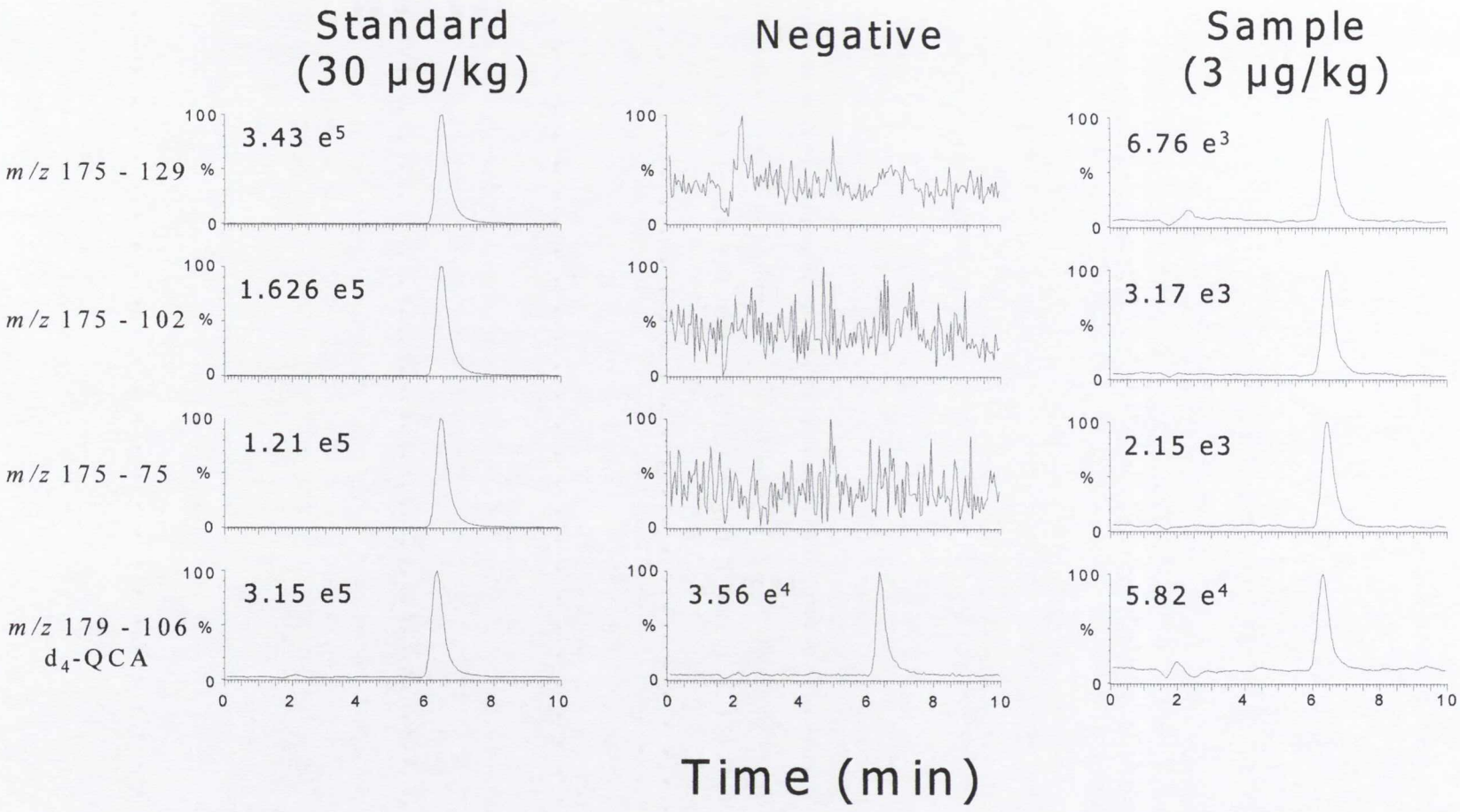

Fig. 3.2. MRM chromatograms of QCA (*m/z* 175 → *m/z* 129, 102 and 75) and d_4-QCA (internal standard; *m/z* 179 → *m/z* 106) in a standard solution (30.0 µg kg^{-1}) of QCA (left column), a known negative liver sample (centre column) and a negative liver fortified with QCA at a concentration of 3 µg kg^{-1} (right column). The high baseline response shown in negative sample (m/z 175 > 129, 175 > 102 and 175 > 106) is due to the magnified scale displayed rather than actual signal.

Table 3.1. **Accuracy and precision of LC-MS-MS method for QCA in fortified porcine liver.**

	3.0 μg kg^{-1}	10 μg kg^{-1}	30 μg kg^{-1}	100 μg kg^{-1}	300 μg kg^{-1}
Day 1					
Mean	3.3	10.0	31.0	98.1	274
s	0.12	1.00	0.44	3.41	3.4
RSD	3.6	1.4	1.4	3.5	1.2
% Recovery	108	103	103	98	91
n	6	6	6	6	6
Day 2					
Mean	3.2	10.3	29.2	88.9	268
s	0.22	0.38	0.68	3.25	10.3
RSD	7.0	3.7	2.3	3.7	3.9
% Recovery	107	103	97	89	89
n	6	6	6	6	6
Day 3					
Mean	3.3	10.4	29.5	89.3	270
s	0.15	0.38	0.65	1.83	5.6
RSD	4.4	3.6	2.2	2.0	2.1
% Recovery	111	104	98	89	90
n	6	6	6	6	6
Overall					
Mean	3.3	10.2	29.9	92.1	271
s	0.17	0.64	0.98	5.13	7.1
RSD	5.1	6.3	3.3	5.6	2.6
% Recovery	109	102	100	92	90
n	18	18	18	18	18

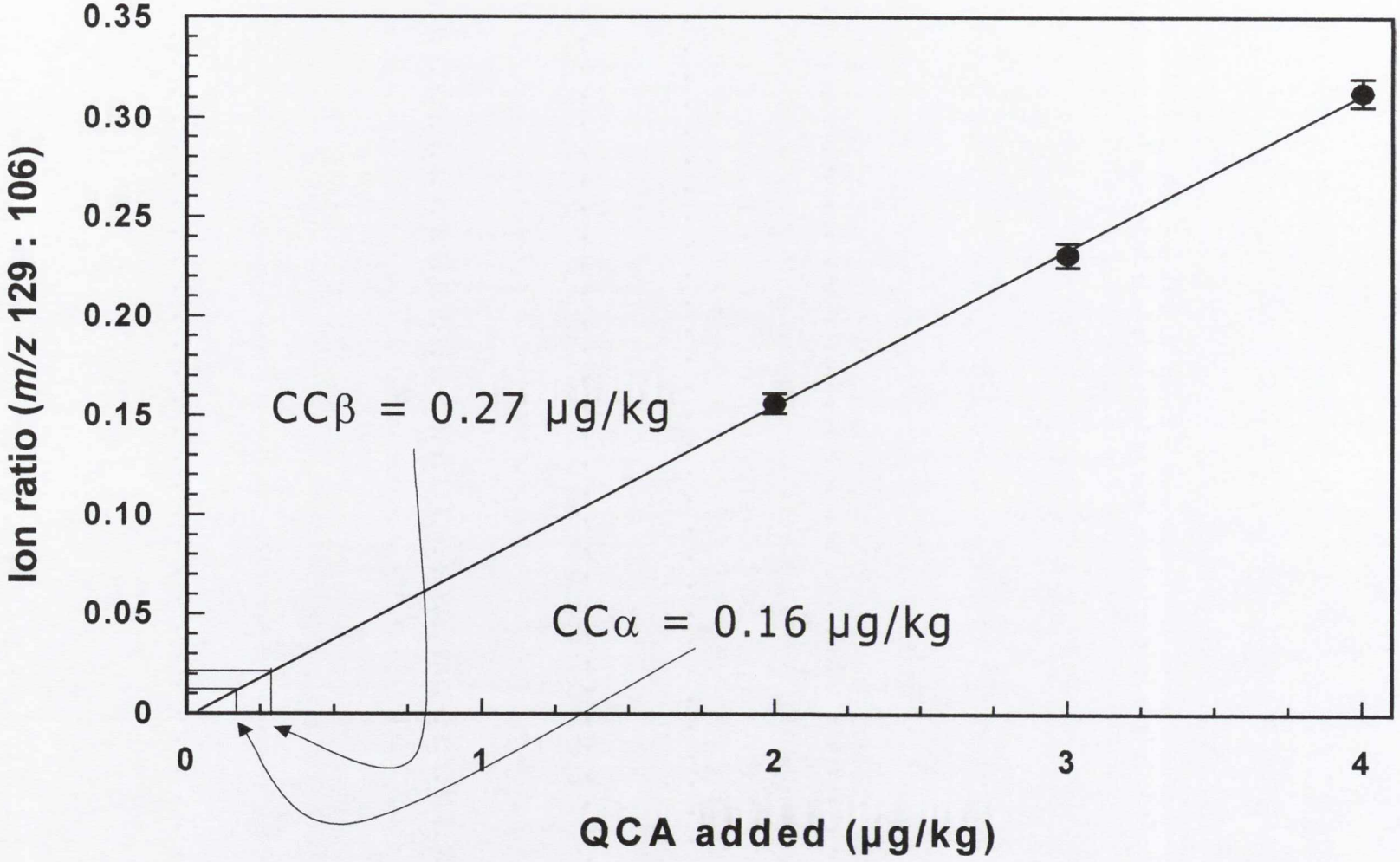

Fig. 3.4. Calculation of CCα and CCβ - graph of signal (*m/z* 129 : 106) *versus* QCA concentration added to known negative liver.

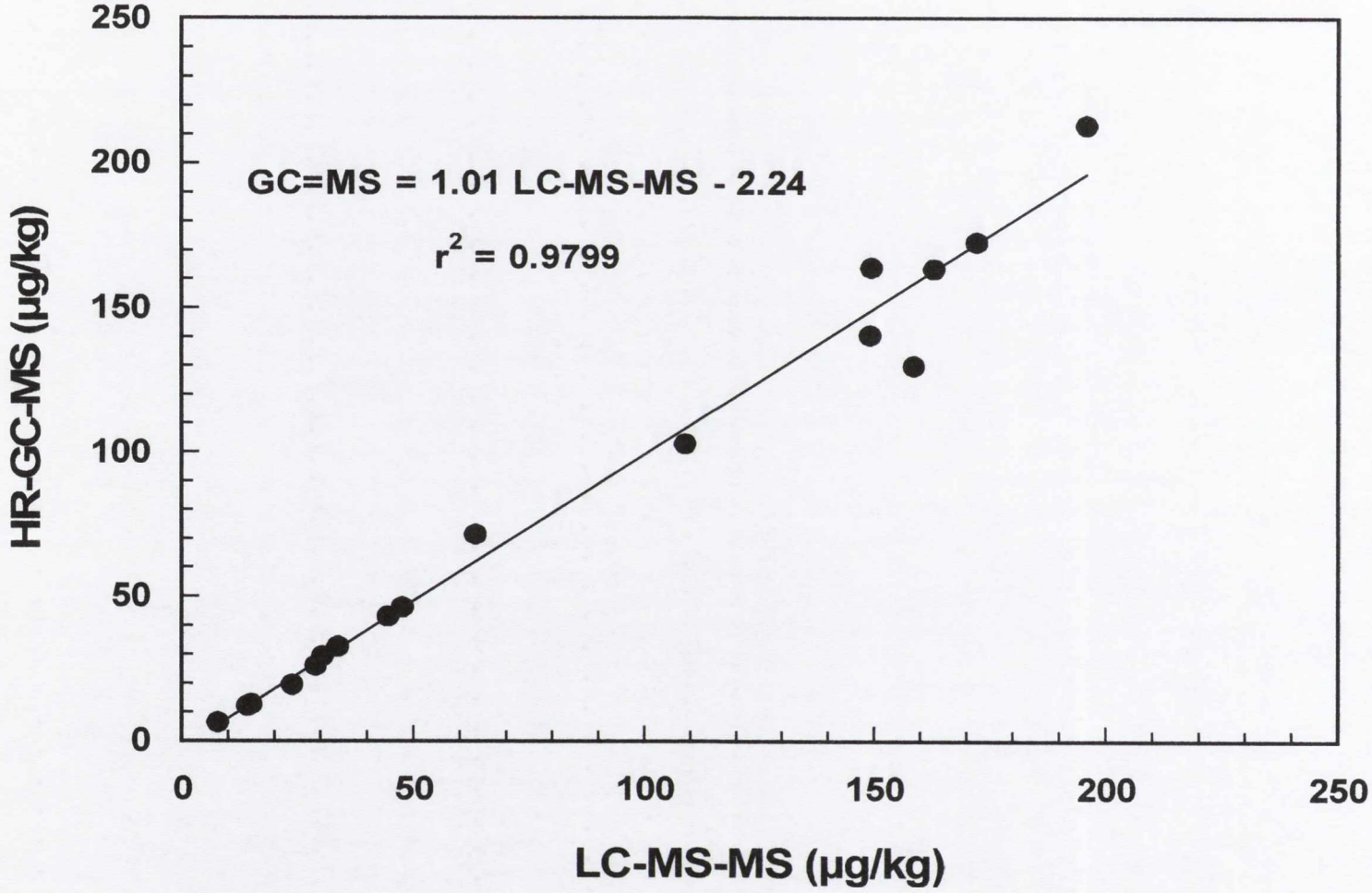

Fig. 3.5. **Correlation between high-resolution GC-MS method for QCA and the described LC-MS-MS assay determined by the analysis of 16 incurred positive porcine liver sample.**

3.3.3. Correlation of the described method with the standard gas chromatography MS method

Sixteen porcine liver samples, which contained incurred QCA residues at concentrations between 6.5 and 213.0 μg kg^{-1}, were selected for analysis using both the GC-MS method and the described method. All of the samples had been collected as part of the statutory residues control schemes in NI. Fig 3.5. shows that the correlation (r^2=0.9799) between the two methods was excellent, confirming the value of the LC-MS-MS method.

3.4. Conclusion

None of the previously published methods for QCA meets the new EC technical criteria for unambiguous confirmation of QCA in tissue, particularly those related to the identification of QCA residues to prevent false positive results. Furthermore, the existing methods are slow, cumbersome and are not applicable to large numbers of samples. We have developed a robust method for the unambiguous confirmation of QCA in porcine tissue. The method has been rigorously validated according to EU Commission Decision 2002/657/EC for use in NI residue control schemes over a relevant concentration range (3.0 – 300 μg kg^{-1}). The linearity of the method has been demonstrated over this concentration range. The performance characteristics CCα and CCβ have been calculated at 0.16 and 0.27 μg kg^{-1}, respectively and the method has been shown to correlate well with the previous in-house GC-MS method.. These show that the described method, which is rapid and capable of handling larger numbers of samples than the classical GC-MS method, is fit for purpose. The method has been routinely applied to the analysis of samples taken in NI under the UK National Surveillance Scheme. The objectives set for this chapter have therefore been fulfilled.

3.5. Publications

The method described in this chapter was published in the *Analyst* in 2002:

Hutchinson, M. J., Young, P. Y., Hewitt, S. A., Faulkner, D., and Kennedy, D. G., 2002, Development and validation of an improved method for confirmation of the carbadox metabolite, quinoxaline-2-carboxylic acid, in porcine liver using LC-electrospray MS-MS according to revised EU criteria for veterinary drug residues. *Analyst*, **127**, 342-346.

Chapter 4

Quinoxaline-2-carboxylic acid in pigs: criteria to distinguish between the illegal use of carbadox and environmental contamination

4.1. Introduction

The objectives of the work presented in this chapter were:

A) To investigate if the CBX metabolite, QCA, can be transferred from a group of medicated animals to a group of unmedicated animals through the use of the same housing, leading to a 'violative' laboratory result, as previously demonstrated with the antimicrobial furazolidone by this laboratory (McCraacken *et al.*, 2000).

(B) If such transfer occurs, to investigate possible ways of differentiating between residues generated due to contamination of the housing and deliberate medication of the animals.

CBX was approved for use as a zootechnical feed additive in the EU, under Council Directive 70/524/EEC in 1974. Over the next 25 years concerns regarding the potential safety of the consumer, the animals and users (operators) were expressed leading the EU to withdraw all marketing authorisations for this compound in 1998. Consequently, there is effectively a zero tolerance for CBX-related residues in food of animal origin. QCA is the most persistent metabolite of CBX and has been designated as the marker residue for CBX use. National Surveillance Schemes for veterinary drug residues are required to monitor for the use of CBX in animals for human consumption. The unequivocal confirmation of the presence of any CBX-related residues should lead to exclusion of affected animals from the human food chain and possible prosecution of the producer in a court of law. However, veterinary drug residues can occur in animals by mechanisms other than deliberate administration. Accidental contamination of animal feeds, as a result of carry-over of medication during the feed milling process from medicated batches of feed to subsequent, ostensibly unmedicated batches of feed has been shown to has been shown to cause a variety of residue problems (see Chapter 1.4.3.) However, this should

not occur with unauthorised compounds, as the feed mills will not have any of these compounds on site. Transfer of drugs may also occur from animal to animal via the passive transfer/ingestion of urine and/or faeces. Even brief exposure to the excretions of previously medicated animals (to mimic transportation to and within lairage of slaughter facilities) has been shown to result in violative residue levels in unmedicated animals (McCracken *et al.*, 2000). This could lead to a producer, whose animals have tested positive for an unauthorised substance and whose animals have correctly been excluded from the human food chain being subjected to further legal proceedings. This leads to the possibility that one producer, presenting contaminated animals, could be further penalised in court as a result of abuse by another producer.

It may therefore be important for National Authorities to have criteria that can assist in the discrimination between contamination and illegal use. Previous work has shown that the use of the ratio concentrations of a compound in both tissue and bodily fluids can be successfully applied to differentiate between these possibilities (McCracken *et al.*, 2000). In such cases of contamination, though it would be appropriate to exclude the carcasses from the human food chain, it would not be necessary to penalise the producer for any offence. The objective of the study was to see if the use of CBX could result in carryover of QCA from treated to untreated pigs. Also if it was subsequently possible to differentiate between environmental contamination and deliberate use by comparison of QCA concentration ratios between body fluids and tissues.

4.2. Experimental

4.2.1 Experiment 1: Depletion of carbadox from pigs

This experiment was carried out to determine the concentrations of QCA in liver, kidney, muscle, urine, and bile in pigs fed CBX at therapeutic levels and then withdrawn from the drug over a period of two weeks. Twenty pigs were kept in a concrete floored house with *ad libitum* access to fresh water. Once a day they were fed a standard pig ration containing the equivalent to 50 mg kg^{-1} CBX (ICN Biomedicals, Oxfordshire, UK) at a rate of 1.0 kg per pig per day over 7 days. After the dosing period, the medicated animals were transferred into clean housing and fed unmedicated rations at the rate of 1.0 kg per pig per day for the remainder of the experiment. On day 0, 4, 7, 11, and 14 post-transfer, four pigs were removed and sacrificed. A further four pigs were killed at the start of the experiment to act as untreated controls. During necropsy, samples of liver (without biliary sack), kidney, muscle, urine and bile were taken. All samples were stored at -20°C prior to analysis.

4.2.2. Experiment 2: Exposure of untreated pigs to carbadox-contaminated housing

This experiment was carried out to compare the concentration of QCA in liver, kidney, muscle, urine and bile of animals exposed to housing contaminated with CBX. The house used for medicating the pigs in Experiment 1 was used as the source of contaminated housing. Following removal of the treated pigs, excess feed along with all faeces and urine was removed by scraping the floor of the house. Thereafter, twenty untreated pigs were transferred into the house. Feed and clean water was available to the pigs at all times. Directly after transfer and at 2, 4, 8 and 24 hours thereafter, 4 pigs were removed and sacrificed. This exceeds any expect exposure time that a group of animals is likely to

experience through transport to, holding and slaughter. At necropsy, samples were removed and stored as in Experiment 1.

4.2.3. Determination of quinoxaline carboxylic acid concentrations in liver, kidney, muscle, urine, and bile.

The method for the detection of QCA in porcine tissues has been described previously (see Section 3.2.). Briefly, each sample was minced and aliquots (5.0 g for solid tissues or 5 ml for fluids) taken for analysis. Deuterated internal standard (d_4-QCA, RIVM, Bilthoven, The Netherlands) was added to each at a concentration of 30 $\mu g\ kg^{-1}$. Samples were hydrolysed in sodium hydroxide (3 mol l^{-1}), acidified and extracted with ethyl acetate. QCA was then back-extracted into phosphate buffer (0.1 mol l^{-1}) at pH 8.0. Sample clean up was performed using automated solid phase extraction (SPE), the eluate being acidified and extracted again with ethyl acetate. All extracts were dried down under a stream of nitrogen and resuspended in methanol-water solution (10 : 90 v/v). QCA in the samples was detected and quantified using LC electrospray MS-MS. This was done by monitoring for transition products derived from the $[M+H]^+$ ion of QCA at *m/z* 129, 102 and 75, and for the transition product derived from the $[M+H]^+$ ion of d_4-QCA at *m/z* 106. All data have been corrected for analytical recovery, as measured by isotope dilution of d_4-QCA, and have been presented as $\mu g\ kg^{-1}$ (tissues) or $\mu g\ L^{-1}$ (fluids) and are shown as Mean ± SEM.

4.3. Results

4.3.1. Experiment 1: Depletion of carbadox from pigs

QCA was not detected in any tissues or fluids taken from the pre-experimental control animals. The depletion of QCA residues in kidney, liver, muscle, bile and urine of pigs fed a therapeutic dose of CBX for 7 days is shown in Fig. 4.1. The QCA concentration was highest in urine before withdrawal at 147.9 ± 64.4 μg kg^{-1} and lowest in muscle (none detected). The QCA concentration in urine, bile, and kidney fell sharply over the 2 week withdrawal period to levels < 2 μg kg^{-1}. Liver concentrations however declined less rapidly and were still easily detectable after 2 weeks at concentrations well in excess of the limit of quantification (LoQ) of the assay at 13.5 ± 2.5 μg kg^{-1}.

4.3.2. Experiment 2: Exposure of untreated pigs to carbadox-contaminated housing.

Within 2 hours of being exposed to contaminated housing, the unmedicated pigs showed detectable concentrations of QCA residues in liver, kidney, bile, and urine (see Fig. 4.2.).

The highest concentrations were observed in urine at (38.5 ± 3.1 μg kg^{-1}) and the lowest in liver (0.8 ± 0.1 μg kg^{-1}). At the end of the 24 hour exposure period, the highest concentrations were again observed in urine (317.2 ± 115.8 μg kg^{-1}) and the lowest in muscle at 0.5 ± 0.3 μg kg^{-1}). Though fluid QCA concentrations increased throughout the exposure period, tissue QCA concentrations remained low. The highest were observed in kidney at 12.1 ± 1.6 μg kg^{-1} after 24 hours exposure. The ranking order (descending) of QCA concentrations was urine > bile > kidney > liver > muscle over the course of the experiment.

4.3.3. Urine : liver quinoxaline carboxylic acid concentration ratio

Pigs treated with CBX and killed without any withdrawal (Experiment 1), had a mean urine : liver QCA ratio of 2.62 ± 2.4 (see Fig. 4.3. B). This ratio fell rapidly over the next 7 days to a mean urine : liver QCA ratio of 0.19 ± 0.24, where it remained for the rest of the withdrawal period. The overall mean QCA urine : liver ratio for pigs subjected to any form of withdrawal (i.e. day 4 to day 14) was 0.31 ± 0.27.

After only 2 hours exposure to contaminated housing, the unmedicated pigs (Experiment 2) had a mean urine : liver QCA ratio of 40.61 ± 13.11 (see Fig. 4.3. A). The mean urine : liver QCA ratio varied over the hour exposure period but was lowest after 24 hours with a mean urine : liver QCA ratio of 10.59 ± 7.74. The overall mean QCA urine : liver ratio for pigs exposed to contaminated housing (i.e. 2 to 24 hours) was 35.51 ± 22.43 (range 4.51-75.29).

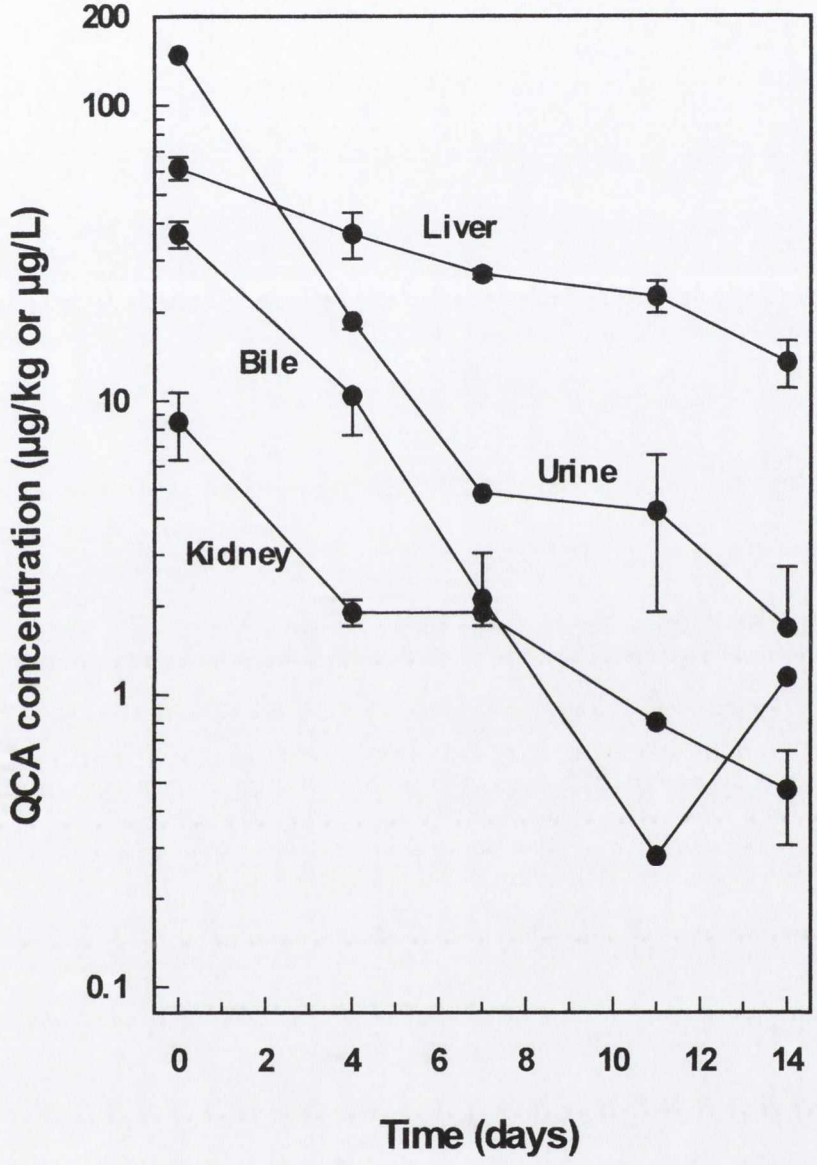

Fig. 4.1. Concentrations of QCA in liver, kidney, muscle, bile and urine for pigs treated with CBX in their diet at a concentration of 50 mg kg^{-1} and withdrawn over a 14 day period.

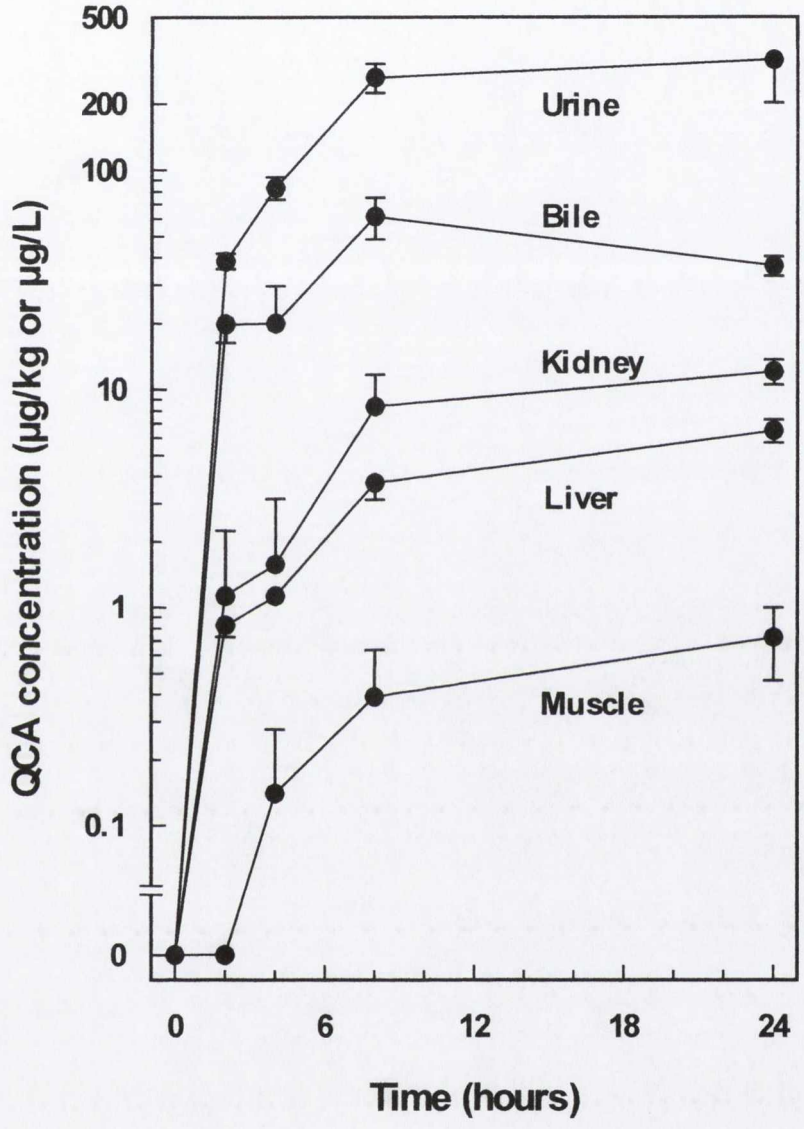

Fig. 4.2. Concentrations of QCA in liver, kidney, muscle, bile and urine for previously unmedicated pigs exposed to housing vacated by CBX treated pigs over a 24 hour period.

4.4. Discussion

Under EU National Surveillance Schemes, detection of any quantity of QCA in a liver sample is a non-compliant result. Commission Regulation EC 2788/98 prohibits the use of CBX in food-producing animals. Previous studies with another unauthorised drug, furazolidone, have shown that it can be transferred from pig-to-pig through even brief contact with accommodation that previously had housed medicated animals. It is desirable therefore to know whether or not it is possible that a non-compliant QCA result could be a result of contact with the urine or faeces of illegally medicated animals, rather than deliberate medication. Additionally, it is desirable to have criteria by which it can be determined whether a non-compliant laboratory result for QCA indicates deliberate abuse or accidental contamination. In order to determine criteria for such a differentiation, the tissues and fluids that were removed from the carcass after sacrifice are all readily accessible for sampling in any commercial processing plant.

4.4.1. QCA in pigs exposed to environmental contamination.

QCA residues are present at detectable levels after 2 hours exposure to a contaminated environment in four of the five tissues taken (see Fig. 4.2.), showing that QCA, as well as being picked up by clean unmedicated pigs, is rapidly absorbed and may be detected in tissues and fluids after short exposure periods. Even such short exposure (2 hours) to a contaminated environment could therefore potentially lead to a non-compliant result in liver and therefore to an offence under EU legalisation.

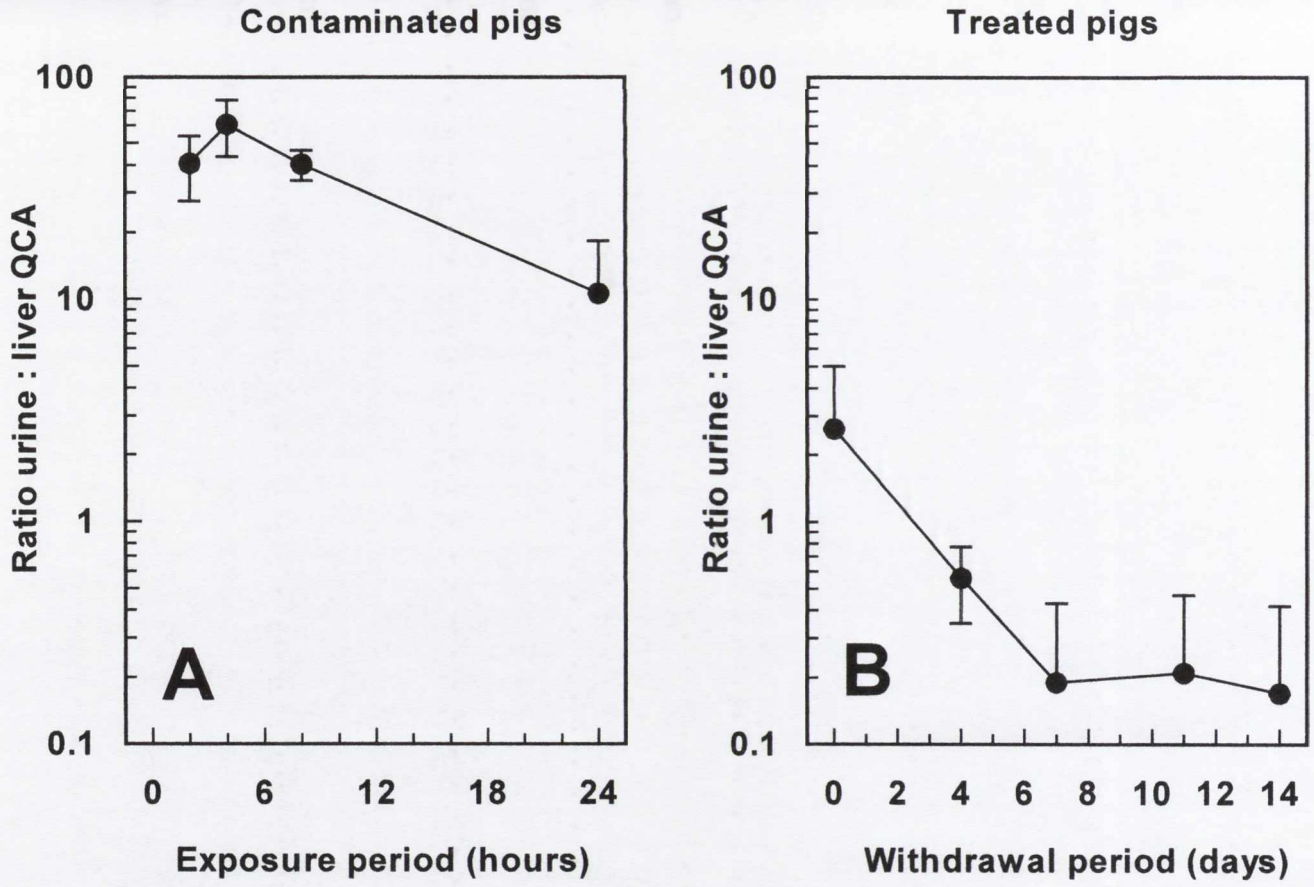

Fig. 4.3. **A . The ratio concentrations of QCA in urine : liver in previously unmedicated pigs exposed to housing vacated by CBX treated pigs. B . The ratio concentrations of QCA in urine : liver in pigs treated with CBX in their diet at a concentration of 50 mg kg^{-1} over a 14 day withdrawal period.**

4.4.2. Possible criteria for distinguishing between abuse and contamination.

The mean urine : liver QCA concentration ratio in animals subjected to medication with CBX and to any form of withdrawal (see Fig. 4.3 B) was 0.56 ± 0.21 (no values greater than 0.8). The mean urine : liver QCA concentration in unmedicated animals, exposed to contaminated housing (see Fig. 4.3 A) was 10.59 ± 7.74 (no values less than 4.5). These figures suggest that it may therefore be possible to differentiate between environmental contamination and deliberate medication on the basis on the urine : liver QCA concentration ratio.

The pharmacokinetics of CBX can be used to explain the difference in these ratios. In an animal subject to medication, the administered CBX undergoes metabolism in the liver to QCA, the liver acts as a 'trap' for QCA and it is slowly removed (over several weeks) from this tissue mainly via bilary excretion. Thus in a deliberately medicated animal, liver concentrations of QCA are high, with lower concentrations in the urine, leading to a low urine : liver ratio. However in a situation where contamination occurs CBX is being passed into the animals system in its already metabolised state as QCA, and therefore does not need to undergo liver metabolism (and is thus not trapped) and can be directly excreted via the kidneys. Therefore in a directly contaminated animal the liver concentrations of QCA are low, with higher urine concentrations leading to a higher urine : liver ratio.

4.4.3. Implications of this study

On the basis of the above results, animals treated with CBX and subject to any withdrawal period would have a urine : liver QCA concentration of less than 0.80, whereas animals

exposed to environmental contamination with CBX would have a urine : liver QCA concentration of greater than 4.5. Any samples falling within the range 0.9 - 4.4 should be therefore considered as inconclusive and be the subject of intensive investigation. Though these are only tentative criteria, based on a small number of animals, they might form the basis of a means of discrimination, whereby competent National Authorities could decide between an offence resulting from deliberate abuse of CBX, and/or exposure of pigs, belonging to an innocent producer, to an environment which had previously contained illegally medicated animals. These criteria are drawn from a small number of samples and may be more accurately redefined by analysis of a larger population.

4.5. Conclusions

Under the EU National Surveillance Schemes, detection of any quantity of QCA in a liver sample constitutes the offence of use of CBX. Previous studies however have shown that drugs can be transferred from pig-to-pig through brief contact with lairage that previously has housed medicated animals. It is desirable therefore to have knowledge of whether it is possible that a violative QCA result could be due to contact with the urine or faeces of illegally medicated animals, rather than deliberate medication. The studies presented in this chapter show that a violative QCA result can be due to exposure to a QCA contaminated environment. It is also desirable to have criteria by which it can be determined whether a positive laboratory test for QCA has been due to deliberate abuse, or as the result of this contact.

The studies presented put forward tentative criteria for the differentiation between exposure to a contaminated environment and deliberate abuse. For ease of laboratory determination, all the tissues and fluids that were removed from the carcass after sacrifice

are all readily accessible for sampling in any commercial processing plant. Thus, assisting competent national authorities to easily set up a testing program to determine whether a positive result for QCA in porcine tissue is truly 'violative'. The objectives set for this chapter have therefore been fulfilled.

4.6. Publications

The work presented in this chapter was accepted by *Food Additives and Contaminants* in 2004 under the title:

Hutchinson, M. J., Young, P. B., and Kennedy, D. G., Quinoxaline-2-carboxylic acid in pigs: criteria to distinguish between illegal use of CBX and environmental contamination.

The results described in this chapter were presented in poster format at the *Fourth International Symposium on Hormone and Veterinary Drug Analysis, Antwerp, Belgium* in 2002 under the same title.

Chapter 5

Simultaneous determination of the carbadox metabolite, quinoxaline-2-carboxylic acid and the olaquindox metabolite, methyl-3-quinoxaline-2-carboxylic acid in porcine liver using liquid chromatography electrospray tandem mass spectrometry

5.1. Introduction

The objectives of the work presented in this chapter were:

(A) To develop a fast and robust confirmatory method for the OQX marker residue in porcine tissue, MQCA, due to the prohibition of the use of OQX in livestock farming.

(B) To validate the developed method as defined by regulations for EU analytical method validation as laid out in EU Commission Decision 2002/657/EC, so the method may be applied to the statutory testing of samples in NI taken under the UK National Surveillance Scheme and Meat Inspection Scheme complying with EU Council Directive 96/23/EC.

OQX is rapidly metabolised in animal tissue via mono and desoxy compounds to MQCA. This is the longest existing detectable metabolite found in tissue and therefore designated as the marker residue for OQX use in animals by the EU. No methods have been described to date for the analysis / conformation of MQCA, only for the detection of OQX or its related desoxy metabolites in tissue. A sensitive method for the extraction and analysis of MQCA residues in animals of food origin was required by the EU. Due to the similarity in chemical structure, the tissue extraction and analysis procedure developed for QCA (see Chapter 3.2.3.) was modified for the dual confirmation of the compounds. We aimed to produce a validated method that could readily confirm QCA and MQCA at concentrations below any anticipated future MRPL that would be set by the European Commission. Samples were then analysed using HPLC linked *via* electrospray interface to tandem MS in positive mode. The EU has revised the criteria that must be applied in both the screening and confirmation of veterinary drug residues in animals of food origin, replacing those previously used. This chapter describes a method for the confirmation of the CBX metabolite, QCA, and the OQX metabolite, MQCA, in porcine liver (the target organ for

analysis) that meets all these new technical criteria. The described method offers a considerable advantage in terms of turnaround time over previously published methods in the numbers of samples that can be processed by a skilled analyst per batch. The accuracy and precision of the method were assessed over a wide concentration range (3 - 150 μg kg^{-1}) to reflect a likely range of naturally incurred samples.

5.2. Experimental

5.2.1. Materials

All solvents were of HPLC grade and all other chemicals were of analytical reagent grade. Distilled or de-ionised water was used throughout the study. A deuterium labelled form of Quinoxaline-2-Carboxylic Acid (d_4-QCA) was obtained from RIVM (European Union Reference Laboratory, Bilthoven, The Netherlands) and QCA was obtained from Sigma-Aldritch Co. Ltd (Gillingham, Dorset, UK.). A deuterium labelled form of Methyl-3-Quinoxaline-2-Carboxylic Acid (d_7-MQCA) was custom synthesised by CSS (Craigavon, Antrim, UK) and MQCA was custom synthesised by QuChem (Queen's University, Belfast, UK.). Stock standard solutions of MQCA (1.0 mg ml^{-1}), d_7-MQCA (1.0 mg ml^{-1}), QCA (1.0 mg ml^{-1}) and d_4-QCA (10.0 μg ml^{-1}) were prepared by dissolving each in methanol. Working standards (1.0 μg ml^{-1}) of all solutions were prepared by serial dilutions of the stock standards in methanol. Stock standards were stable for 1 year and working standards were stable for at least 3 months when stored in amber vials below 4 °C.

Protease Type XIV for enzymatic digestion was obtained from Sigma-Aldritch Co. Ltd (Gillingham, Dorset, UK.). Solutions of protease were prepared in water (50 mg ml^{-1}) and

remade each day, when required. The enzyme digest solution was a buffer solution made up of 0.2 M Tris(hydoxymethyl)methylamine and 0.1 M calcium chloride at pH 9.6 (TRIS/CaCl buffer). The back extraction solution was 0.1 M sodium phosphate buffer, pH 8.0. Mobile phase A (A) contained methanol-acetonitrile-water-acetic acid (10 : 10: 79.6 : 0.4 v/v) and mobile phase B (B) contained pure methanol. Both were filtered and degassed before use.

5.2.2 Equipment

Electrospray LC-MS-MS System

Tandem MS analyses were performed using a Quattro LC (Micromass, Wythenshawe, UK) in positive mode. A Hewlett Packard (Stockport, Cheshire, UK) HPLC system comprising an 1100 Series binary pump, autosampler and solvent degasser were coupled *via* electrospray interface to the Quattro LC. Two Luna 3µ C_{18} 150 X 2.0 mm (Phenomenex, Macclesfield, Cheshire, UK) LC columns were used each with a 3µ 2.0 mm C_{18} guard column (Phenomenex, Macclesfield, Cheshire, UK) attached. The MS source was maintained at 150 °C with nitrogen used as the drying and nebulising gas at a flow rate of 600 and 80 l h^{-1}, respectively. The sample volume injected was 15 µl and the total run time was 20 min. The mobile phase flow rate was 0.2 ml min^{-1} with all the column effluent entering the mass spectrometer.

Before beginning analysis, both columns were equilibrated by pumping mobile phase at pre-run conditions for 30 - 45 min. A gradient programme was run for each sample, the pre-run conditions were 100 : 0 (mobile phase A : B). After 1 min, this was ramped to 20 : 80 (mobile phase A : B) over 9 min and then held until 15 min when the mobile phase was returned to pre-run conditions 100 : 0 (mobile phase A : B) over 0.5 min and held there

until the end of the run at 20 min. Each column was attached to the MS system *via* a switch valve (Prolab, England, UK), while one column was in use its companion was re-equilibrated to pre-run mobile phase conditions.

5.2.3. Sample Extraction

Aliquots of homogenised test or control liver (5.00 ± 0.05 g) were weighed into polyethylene tubes (50 ml). Two known negative samples were analysed in every batch. In addition, four recovery samples, fortified at 10.0 μg kg^{-1} were prepared by the addition of both QCA and MQCA working standard solution (50 μl) to known negative tissue. A further two check samples were analysed with every batch. These were known negative samples that had been fortified with QCA and MQCA at concentrations unknown to the analyst. Fortified samples for validation purposes were also prepared at this stage by addition of the relevant amount of QCA or MQCA standard solutions. Internal standards, d_4-QCA and d_7-MQCA (50 μl of the 1.0 μg ml^{-1} working standards) were added to every sample to be analysed. To all samples TRIS/CaCl buffer (9 ml) and protease solution (50 μl, 50 mg ml^{-1}) was added, the contents mixed for 30 sec., and incubated at 55 °C overnight. Samples were removed and allowed to cool to room temperature and then centrifuged (2000*g*, 4 °C, 5 min.). The supernatant was poured off and to this concentrated hydrochloric acid (1 ml) added. The subsequent ethyl acetate extraction, phosphate buffer extraction, SPE and further ethyl acetate extraction of QCA and MQCA from tissue matrix are identical to those in the QCA extraction method stated in Chapter 3 from this stage onwards (see Chapter 3.2.3.). The extraction steps are outlined briefly below.

Ethyl acetate (6 ml) was added and tubes shaken and centrifuged (2,000g, 4 °C, 10 min.) The upper layer was transferred into a polyethylene tube (50 ml) and the extraction repeated. Back extraction solution (0.1 mol l^{-1}, pH 8.0, 8 ml) was added to the extract, the tubes shaken and centrifuged (2000g, 4 °C, 10 min.). The upper organic layer was aspirated to waste and an aliquot (4 ml) of the aqueous phase transferred to a 10 ml glass tube containing concentrated hydrochloric acid (1 ml). The SPE was carried out on an ASPEC XL4 coupled to a 404-syringe pump (Gilson Ltd, Middleton, Wisconsin, USA) using non-endcapped benzenesulphonic acid (SCX), 1 g sorbent material, 3 ml capacity, disposable solid phase extraction cartridges (IST Ltd, Mid. Glamorgan, UK). Each eluted sample was acidified with concentrated hydrochloric acid (300 µl). Ethyl acetate (2 ml) added, the tubes mixed and centrifuged (2000*g*, 4 °C, 10 min.). The upper organic layer was transferred into 6 ml tubes. The extraction was repeated a further two times with the extracts being combined. The sample extracts were taken to dryness under a stream of nitrogen at 60 °C, methanol-water solution (10 : 90 v/v, 100 µl) added and tube vortexed for 15 sec.

Standards were prepared at 10 µg kg^{-1} by the addition of QCA, MQCA, d_4-QCA and d_7-MQCA working standards (10 µl, 1 µg ml^{-1}) to 3 ml glass tubes and taken to dryness under nitrogen. Methanol-water solution (10 : 90 v/v, 100 µl) was added and the tube vortexed. All the solutions were then transferred to tapered microvials for analysis.

5.2.4. LC-MS-MS analysis

Spectra for MQCA and D_4-MQCA were obtained over the range *m/z* 50-200 with the instrument configured for MS only; spectra for QCA and d_4-QCA have been previously

shown in Chapter 3.3.1. Multiple reaction monitoring (MRM) was set for the detection of QCA, d_4-QCA, MQCA, and d_7-MQCA. Quadrupole 1 was set to transmit the molecular ions ($[M + H]^+$) of QCA (*m/z* 175), d_4-QCA (*m/z* 179), MQCA (*m/z* 189) and d_7-MQCA (*m/z* 196). Quadrupole 2 was set to transmit the QCA product ions at *m/z* 102 and 75, the MQCA product ions at *m/z* 145 and 102 along with the internal standard product ions for d_4-QCA and d_7-MQCA at *m/z* 106 and 152, respectively. Argon was used as the collision gas and was bled into the cell at a pressure of 2.3×10^{-3} mbar. The collision cell entrance and exit energies were set at 0 and 2 eV, respectively. The collision energy for the QCA product ions at *m/z* 102 and 75 were optimised at 30 and 45 eV, respectively. The collision energy for the MQCA product ions at *m/z* 145 and 102 were optimised at 16 and 34 eV, respectively. The internal standard product ions for d_4-QCA (*m/z* 106) and d_7-MQCA (*m/z* 152) were optimised at 30 and 16 eV, respectively. The cone voltages for QCA and MQCA were 30 and 20 V, respectively, and the dwell time for each ion was 0.5 sec.

Concentrations for QCA were calculated by comparing the ratio of the *m/z* 175 → *m/z* 102 (QCA base peak) response with the *m/z* 179 → *m/z* 106 response (d_4-QCA) in the sample with those in the standards (10.0 μg kg^{-1}) within the run. Concentrations for MQCA were calculated by comparing the ratio of the *m/z* 189 → *m/z* 102 (MQCA base peak) response with the *m/z* 196 → *m/z* 152 response (d_7-MQCA) in the sample with those in the standards (10.0 μg kg^{-1}) within the run.

5.3. Results and discussion

5.3.1. Tandem MS analysis of quinoxaline carboxylic acid and methyl quinoxaline carboxylic acid

The fragmentation pattern of QCA has already been described in Chapter 3 (see Fig 3.1.). The MS-MS of the molecular ion of QCA (*m/z* 175) produces a prominent product ion at *m/z* 129. This results from the successive loss of water and carbon monoxide supported by the observation of a small peak at *m/z* 157 – the loss of H_2O. Similarly, the MS-MS of the molecular ion of MQCA (*m/z* 189) also produces a prominent product ion resulting from the successive loss of water and carbon monoxide (*m/z* 145). This again is supported by the observation of a small peak (*m/z* 171) resulting from the loss of H_2O (see Fig. 5.1.). An identical product ion for QCA and MQCA is formed at *m/z* 102, in the case of QCA it is due to the loss of HCN from the *m/z* 129 product. For MQCA it is due to the loss from the *m/z* 145 product ion of a methyl group (CH_3) followed by HCN. QCA produces another prominent product ion at *m/z* 75; this can be attributed to the successive loss of another HCN from the *m/z* 102 product. MQCA shows a similar loss of HCN that is supported by the observation of a prominent peak at *m/z* 75.

According to draft technical criteria for residue identification in food of animal origin, a maximum of 4 identification points (IPs) are required to confirm unauthorised substances (see Chapter 1.11.). The criteria score 1.5 IPs for each tandem MS product ion measured, plus 1 IP for the precursor ion whether it is measured or not. The described method scores 4 IPs for both QCA and MQCA through the measurement of two product ions (plus one precursor ion). Both thus fulfil the identification criteria. However, for unambiguous identification the ion ratios of unknown samples have also to correspond to those in the standards within the run, within predefined limits.

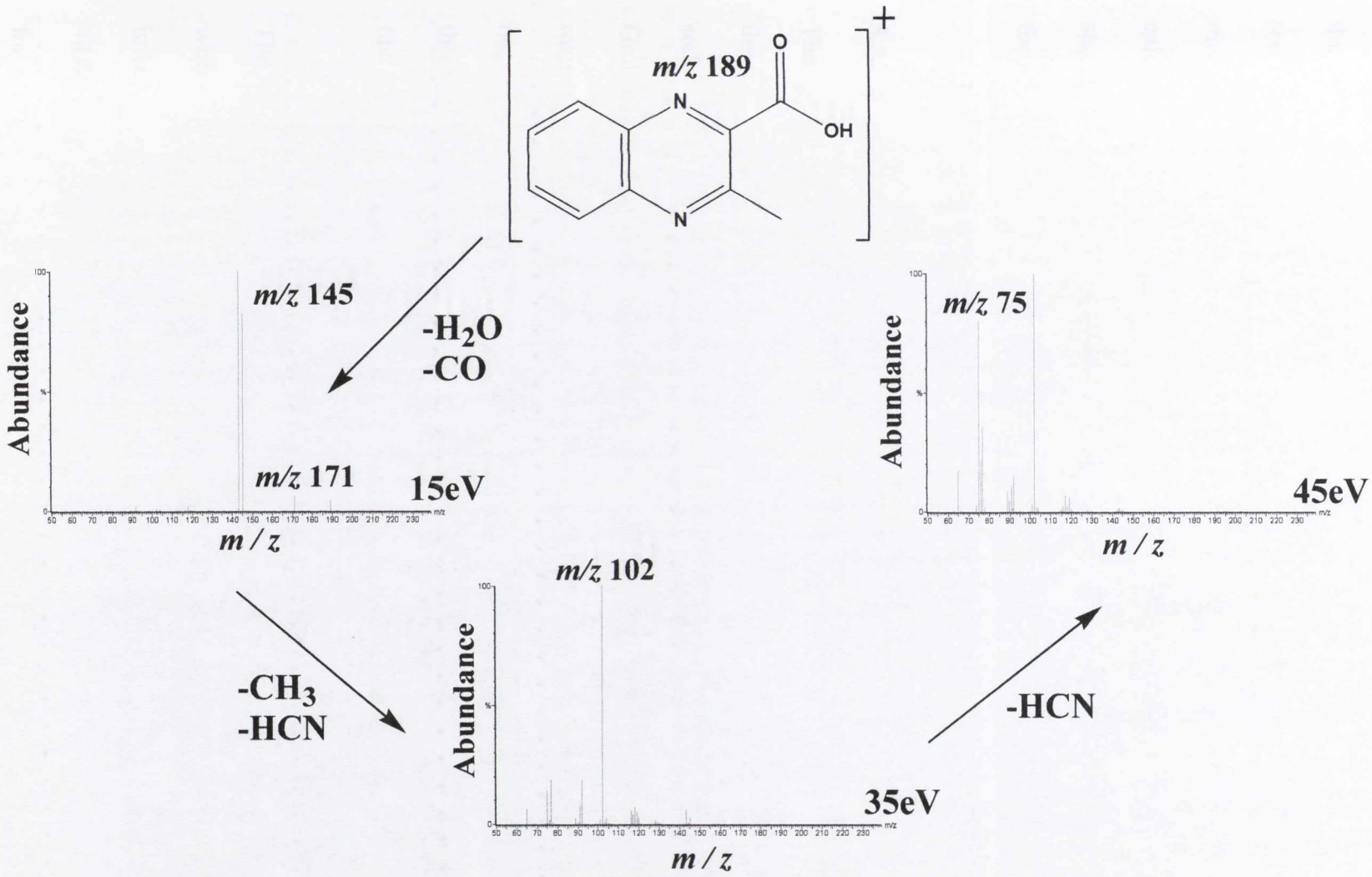

Fig. 5.1. MS-MS spectrogram of the molecular ion $[M + H]^+$ of MQCA at *m/z* 189.

The tolerances allowed for the ion ratios vary relative to the intensity of the product ion to the base peak ion. For QCA the ion ratio of *m/z* 75 : 102 was measured and for MQCA the ion ratio of *m/z* 102 : 145 was measured. These ratios had to meet the previously mentioned pre-set tolerances before they were considered acceptable All results were calculated by comparison of the ratio of the analyte response to the equivalent internal standard response in the sample with those in the bracketed standards (10.0 μg kg^{-1}) within the run.

5.3.2. Method performance characteristics

The extraction method differs from that described in Chapter 3. To allow the inclusion of the deuterated internal standard for MQCA (d_7-MQCA), it was necessary to remove the sodium hydroxide hydrolysis step and replace it with a protease digest as used by Van Ginkel *et al.* (1995). The rest of the extraction technique remained the same; this had the major advantage of permitting the processing of 16 samples in duplicate (excluding negatives, controls and check samples) in 1.5 days by a skilled analyst. Modification of this method now allows the extraction and analysis of MQCA as well as QCA using this fast and robust procedure.

The absolute recovery (that is based on the analysis of 4 negative liver samples fortified with both QCA and MQCA at 10 μg kg^{-1} and carried though the method in the absence of internal standard) achieved by the described method is 54.2 ± 0.9 % (Mean ± *s*, n = 4) and 48.0 ± 2.9 % (Mean ± *s*, n = 4) for QCA and MQCA, respectively. The recovery achieved for analysis of QCA using the reported dual method is comparable to the value previously obtained for the singular analysis of QCA (57.6 ± 5.1 %). Fig. 5.2. shows MRM chromatograms for a QCA standard (10 μg kg^{-1}), a negative liver and a negative liver

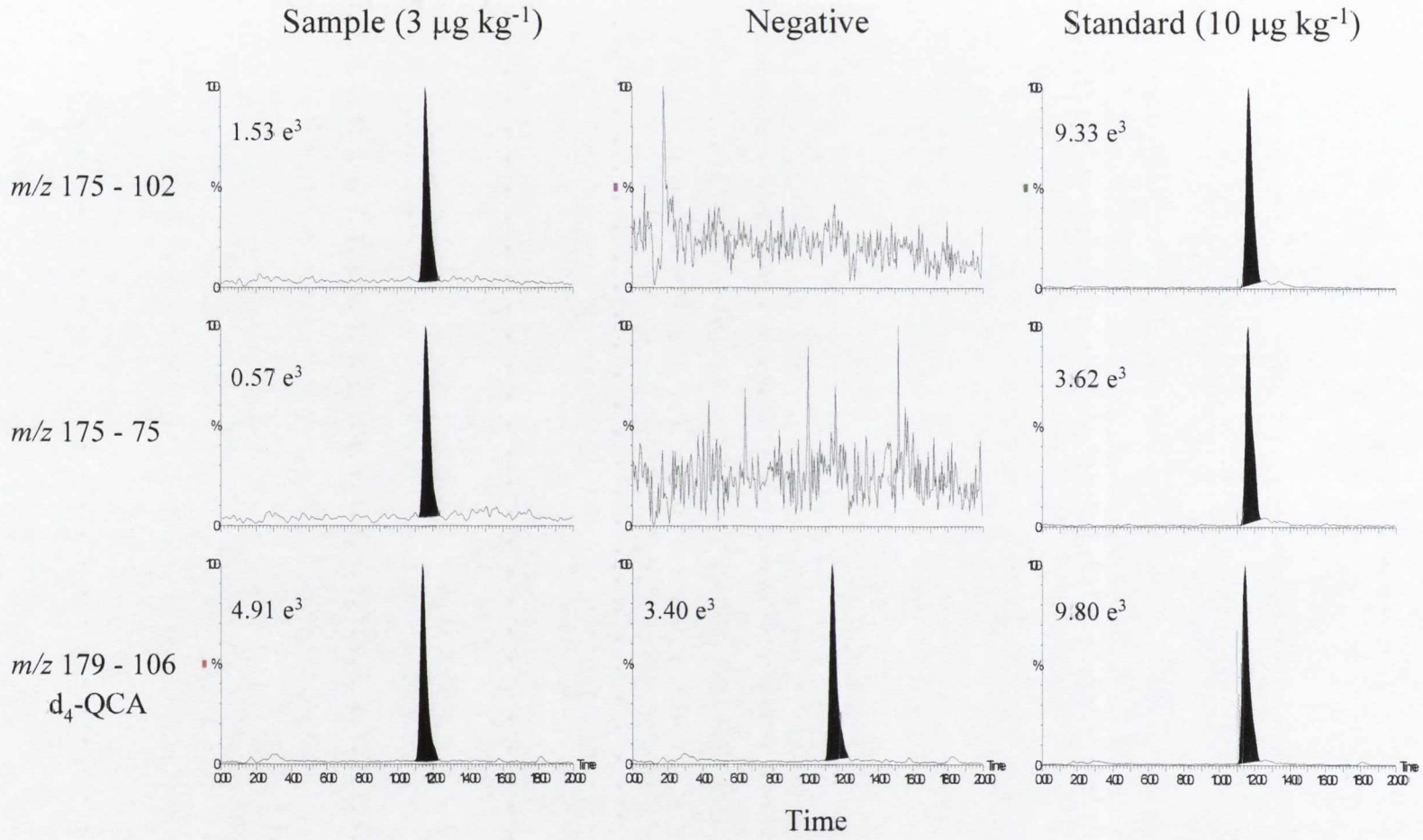

Fig. 5.2. MRM chromatograms of QCA (*m/z* 175 → *m/z* 102 and 75) and d_4-QCA (internal standard; *m/z* 179 → *m/z* 106) in a negative liver fortified with QCA at a concentration of 3.0 μg kg^{-1} (left column), a known negative liver sample (centre column) and a standard solution (10.0 μg kg^{-1}) of QCA (right column),

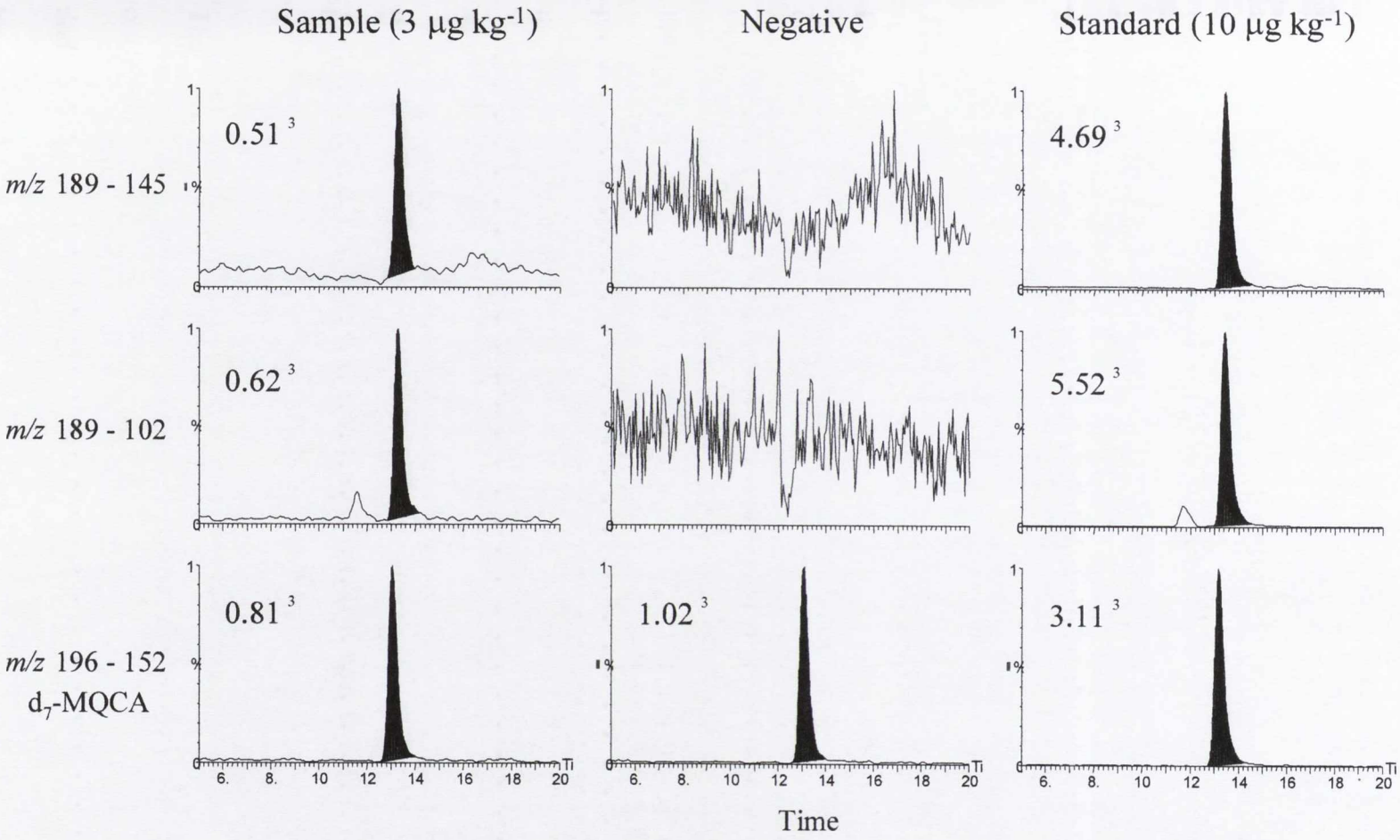

Fig. 5.3. MRM chromatograms of MQCA (m/z 189 → m/z 145 and 102) and d_7-MQCA (internal standard; m/z 196 → m/z 152) in a negative liver fortified with MQCA at a concentration of 3.0 μg kg^{-1} (left column), a known negative liver sample (centre column) and a standard solution (10.0 μg kg^{-1}) of MQCA (right column).

fortified with QCA at 3.0 μg kg^{-1} at *m/z* 102, 75 and 106 (internal standard). Fig. 5.3. shows MRM chromatograms for a MQCA standard (10 μg kg^{-1}), a negative liver and a negative liver fortified with MQCA at 3.0 μg kg^{-1} at *m/z* 145, 102 and 152 (internal standard). Both sets of chromatograms showed no spurious peaks due to the matrix. The negative samples for both sets of chromatograms show only the internal standard peak (*m/z* 106 and 152, QCA an MQCA, respectively) with only background noise spectra present for each of the other ions monitored. Thus showing the blank samples are truly negative.

The accuracy and precision of the method was determined over the concentration range (3.0 - 150 μg kg^{-1}) on three separate occasions, which reflects QCA concentrations encountered in NI statutory control schemes, and also provides a wide concentration range over which to assess MQCA method performance (see Table 5.1. and Table 5.2.). Over the assessed concentration range, the results displayed the same satisfactory accuracy and precision as the previously published method. QCA shows over this concentration range a linear relationship between the signal (ratio of the transition *m/z* 175 → 102 to transition *m/z* 179 → 106) and fortified concentration ($r^2 = 0.998$) (Data not shown). The same linear relationship is also observed for MQCA between signal (ratio of transition *m/z* 189 → 102 to transition *m/z* 196 → 152) and fortified concentration ($r^2 = 0.952$) (Data not shown).

The European Commission has set out new procedures for the determination of a confirmatory methods analytical limit. This involves the calculation of the Decision Limit and Detection Capability. The Decision Limit (CCα) is defined as the limit at which it can be decided that a result is violative with an error probability of α (α = 1% for unauthorised substances). The Detection Capability (CCβ) is defined as the smallest concentration of

analyte that can be identified and quantified in a sample with a statistical certainty of 1-β (β ≤ 5% for unauthorised substances). CCα and CCβ were calculated for both QCA and MQCA, each result having to fulfil all EU criteria for residue identification, before it could be considered for use. These analytical limits may be determined by the analysis of replicate blank samples (n = 6), fortified at 1.0, 1.5 and 2.0 times the minimum required performance limit (MRPL) - a limit established by the EU, designed to harmonise analytical methods for unauthorised substances in the EU. However, no MRPL for either QCA or MQCA has yet been set.

Therefore, we have chosen to assess CCα and CCβ using a lower MRPL (2.0 μg kg^{-1}) than that is expected to be set for either compound, to ensure that the method will meet the level finally adopted within the EU. Replicate fortified blank samples (n = 6) were prepared at 2.0, 3.0 and 4.0 μg kg^{-1} for QCA and MQCA. A graph of signal ratio (m/z 175 → 102 : m/z 179 → 106 for QCA and m/z 189 → 102 : m/z 179 → 152 for MQCA) versus added concentration was constructed for both compounds. CCα was calculated as the concentration corresponding to the Y intercept plus 2.33 times the standard error of the Y intercept. CCβ was calculated as the concentration corresponding to the Y intercept for CCα plus a further 1.64 times the standard error (see Fig. 5.4. and Fig. 5.5.). The Decision Limit (CCα), the lowest concentration that can be distinguished from zero with an α error probability of 0.01, was calculated at 0.34 μg kg^{-1} and 0.82 μg kg^{-1} for QCA and MQCA, respectively. The Detection Capability (CCβ), the point where 95% of samples will be declared violative with a β error probability of 0.05, was calculated at 0.57 μg kg^{-1} and 1.40 μg kg^{-1} for QCA and MQCA, respectively.

Table 5.1. Accuracy and precision of the LC-MS-MS method for QCA in fortified porcine liver.

	3.0 μg kg^{-1}	10.0 μg kg^{-1}	50 μg kg^{-1}	150 μg kg^{-1}
Day 1				
Mean	3.2	10.2	47.3	137.6
s	0.1	0.2	0.7	2.7
RSD	1.9	1.9	1.4	2.0
% Recovery	106.2	101.7	94.6	91.7
n	6	6	6	6
Day 2				
Mean	3.2	10.7	50.1	143.4
s	0.3	0.4	2.4	4.1
RSD	10.0	4.1	4.2	2.1
% Recovery	107.9	106.7	100.3	95.6
n	6	6	6	6
Day 3				
Mean	3.2	9.7	48.5	144.2
s	0.2	0.3	1.2	2.7
RSD	6.9	2.8	2.4	1.9
% Recovery	107.5	96.9	97.1	96.2
n	6	6	6	6
Overall				
Mean	3.2	10.2	48.7	141.8
s	0.2	0.5	1.8	4.0
RSD	6.7	5.0	3.7	2.8
% Recovery	107.2	106.3	97.3	94.5
n	18	18	18	18

Table 5.2. Accuracy and precision of the LC-MS-MS method for MQCA in fortified porcine liver.

	3.0 μg kg^{-1}	10.0 μg kg^{-1}	50 μg kg^{-1}	150 μg kg^{-1}
Day 1				
Mean	2.8	10.6	50.4	141.8
s	0.2	0.8	4.3	7.3
RSD	7.9	7.1	8.6	5.2
% Recovery	93.8	106.4	100.8	94.6
n	5	6	6	6
Day 2				
Mean	3.6	10.8	51.2	144.4
s	0.3	0.5	2.6	3.9
RSD	8.9	5.1	5.2	2.7
% Recovery	119.7	108.3	102.4	96.3
n	6	6	6	6
Day 3				
Mean	3.3	10.5	48.0	132.1
s	0.4	0.4	0.6	4.3
RSD	3.9	4.0	1.2	3.3
% Recovery	118.5	105.2	96.1	88.1
n	6	6	6	6
Overall				
Mean	3.3	10.7	49.9	139.4
s	0.4	0.6	3.1	7.5
RSD	12.9	5.4	6.2	5.3
% Recovery	111.2	106.6	99.7	90.2
n	17	18	18	18

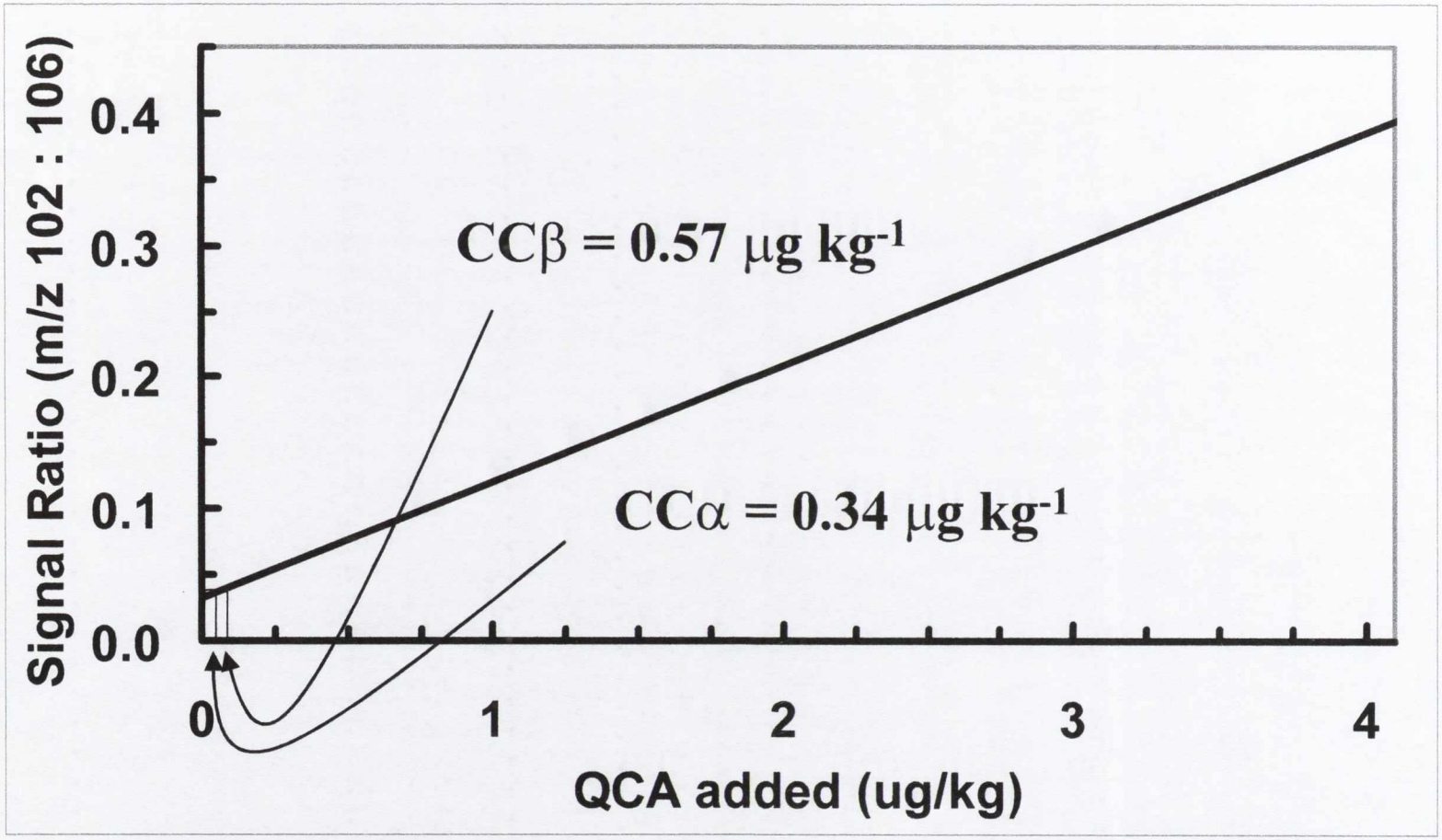

Fig. 5.4. Calculation of CCα and CCβ - graph of signal (*m/z* 102 : 106) *versus* QCA concentration added to known negative liver.

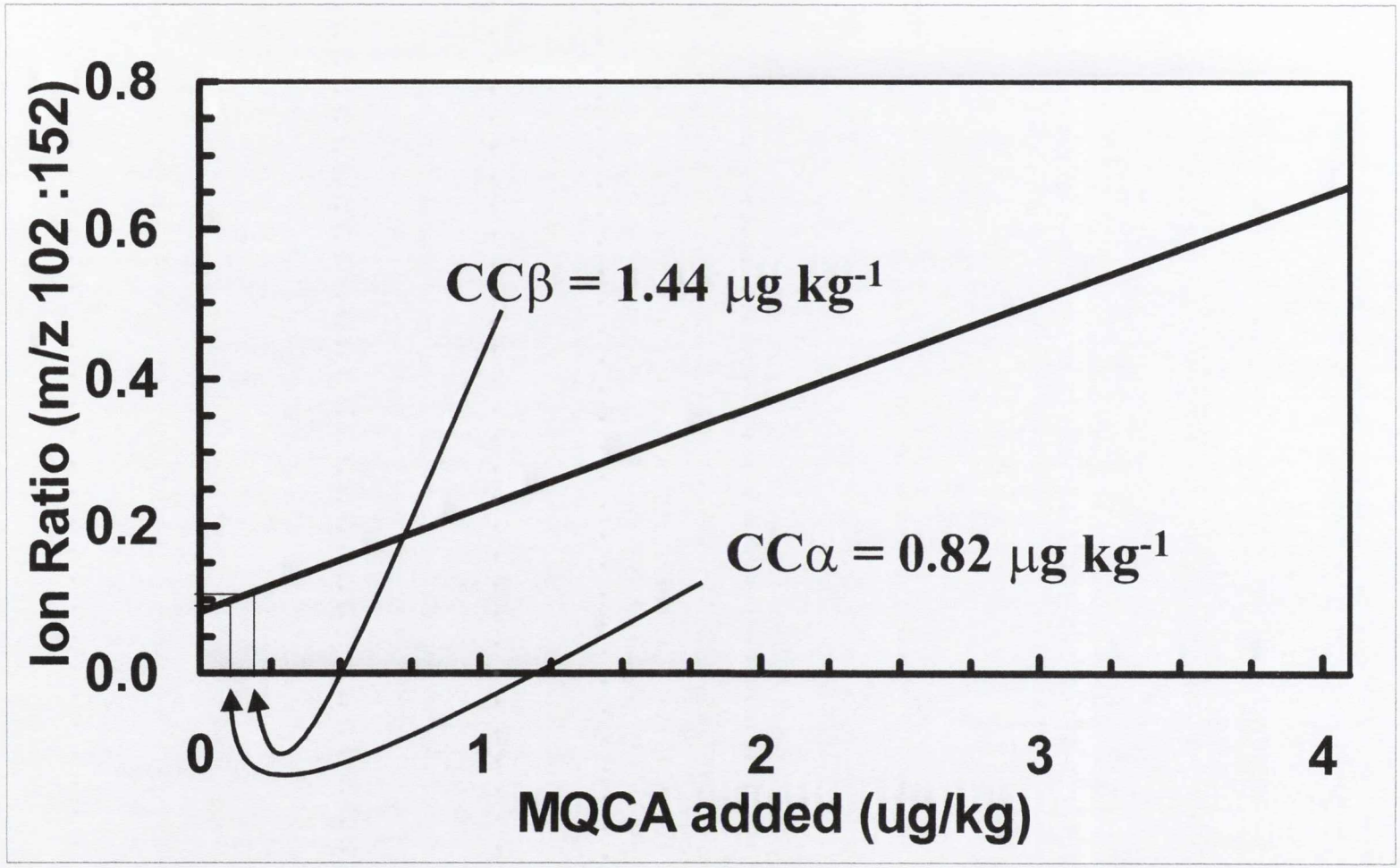

Fig. 5.5. Calculation of CCα and CCβ - graph of signal (m/z 102 : 152) versus MQCA concentration added to known negative liver.

To assess the ability of the method to work with possible field samples, incurred liver samples from pigs fed OQX medicated feed (10 μg kg^{-1}, one tenth of the recommended prophylactic dose) for 4 days, were tested for the presense of MQCA. MQCA residues were detected at significant levels (~16 μg kg^{-1}) by this method (data not shown).

5.4. Conclusions

A fast and robust method has been developed for the detection, confirmation, and quantification of both QCA and MQCA, the marker substances for the use of CBX and OQX, respectively, in porcine tissue. The method has been validated according to EU Commission Decision 2002/657/EC. All results before they are considered viable for use were assessed using new EU criteria for the unambiguous confirmation of prohibited substances. The linearity, accuracy and precision have been demonstrated over the concentration range 3.0 – 150 μg kg^{-1}, and the performance characteristics (CCα and CCβ) have been calculated for the described method. Although an MRPL for QCA or MQCA has not yet been set, the method performance characteristics were assessed at a lower MRPL value (2.0 μg kg^{-1}) than is expected to be set for both compounds, thus ensuring the method will meet any concentration finally adopted by the EU.

The method meets all EU criteria for method validation and is therefore suitable for use in the unambiguous confirmation of both QCA and MQCA in porcine tissue. This method has been routinely applied to the analysis of samples taken in NI under the UK National Surveillance Scheme. The objectives set for this chapter have therefore been fulfilled.

5.5. Publications

The method presented in this chapter has been submitted to the *Journal of Chromatograhy B* in 2003 under the title:

Hutchinson, M. J., Young, P. B., and Kennedy, D. G., Development and validation of a dual method for the confirmation of quinoxaline-2-carboxylic acid (a metabolite of carbadox) and methyl-quinoxaline-2-carboxylic acid (a metabolite of olaquindox), in porcine liver using LC-electrospray MS-MS.

Chapter 6

Summary of Thesis

6.1. Introduction

There is public concern that the widespread use of veterinary drugs in agricultural practice will give rise to the presence of harmful residues in animal tissues or products destined for human consumption. Acute cases of pharmacological toxicity due to β-agonist drugs and β-lactam antibiotics have been reported in Europe. Concerns have also been raised over the carcinogenic and genotoxic nature of some drugs and/or their metabolites commonly used in livestock and poultry production. Since the mid. 1970s, the EU, in partnership with other trading blocks and unaffiliated countries, has introduced legislation governing the use of veterinary drugs and the monitoring of their residues. This is to guarantee the safety of the consumer as well as assuring the quality of imported and/or exported products. MRLs have been set for currently licensed drugs and zootechnical feed additives, with an effective 'zero tolerance' policy for residues of illegal drugs or compounds that have had their product licence withdrawn. Each Member State is required to set up its own National Surveillance Scheme to police these set MRLs and identify misuse. This requires the screening of thousands of samples per year with the subsequent confirmation of any samples suspected to be in breech of EU regulations. Ideally, all Member States should be able to test for all the licensed drugs and illegal substances laid out in EU legislation. Unfortunately, analytical methods for the confirmation of every drug currently used in modern veterinary medicine do not exist, thus, until such a time Member States should show that they have exercised 'due diligence'. Confirmatory methods must be developed by NRLs and CRLs to comply with current legislation, conduct pharmacokinetic studies, and investigate persistent residue problems. Such methods should be based on molecular spectrometry as it allows unambiguous compound identification.

The quinoxaline-N-dioxides, when administered as feed additives can significantly improve feed conversion efficiency and slaughter weight in pigs. They have also been found effective against a range of gram negative gastrointestinal infections. In the 1980s and 90s an increasing body of evidence suggested that such compounds were carcinogenic, mutagenic and in the case of some derivatives, highly phototoxic. This led to the prohibition of CBX and OQX as zootechnical feed additives in 1998 by the European Commission. Several methods are presented in this thesis for the unambiguous confirmation of the banned quinoxaline-N-dioxides, CBX, and OQX in animal feedingstuffs and in porcine tissue. Liquid chromatography linked *via* electrospray interface to tandem MS was used in the analysis of both compounds to provide the sensitivity and specificity needed to meet new EU legislation for the confirmation of veterinary drugs.

6.2. Confirmation of carbadox and olaquindox in feeds

Though CBX and OQX are prohibited within the EU, they are still licensed for use as feed additives in other non-EU countries and are available as premixed zootechnical feed products, thus, the potential still exists for illegal use of both drugs within Europe. It has been demonstrated that low concentrations of drugs can be carried over from one batch of medicated feed into several subsequent batches of unmedicated feed causing unwanted and illegal residue levels in tissue. A current requirement within the EU is for a confirmatory method capable of determining low concentrations of CBX and OQX in porcine animal feed, thus aiding the feed industry to spot sources of contamination and eliminate them, and allowing Member States to police the ban.

The main objective of Chapter 2 was therefore to develop a dual confirmatory method for CBX and OQX in porcine feedingstuffs that met all current EU criteria for method validation. This method employs a simple chloroform-acetonitrile (50 : 50 v/v) extraction, with no additional clean-up to separate the analytes from the feed matrix. Matrix based standards are employed, with both samples and standards separated chromatographically *via* HPLC using an isocratic mobile phase of acetonitrile-water-formic acid (17.5 : 82.4 : 0.1 v/v). The HPLC system is connected *via* electrospray interface to the MS where analysis is carried out by tandem MS in positive mode. The MS-MS of the CBX and OQX molecular ions $[M + H]^+$ at *m/z* 263 and 264, respectively, produces daughter ions at *m/z* 231 and 89 for CBX and *m/z* 212 and 143 for OQX. The base peak ion at *m/z* 231 and *m/z* 212 for CBX and OQX, respectively, are used to calculate the concentrations of each drug in the sample by comparison to the same ion in the matrix standards within the run. The method was validated according to current EU criteria for confirmatory methods. The validation was carried out at 1 – 10% of the EU's prior legal infeed concentration (0.5 – 5.0 mg kg^{-1}) to reflect the necessity of detecting possible contamination levels of the drug in compound feedingstuffs. The Limit of Determination for this method is 0.5 mg kg^{-1} as this is the lowest concentration at which the described method has been validated with a specified degree of accuracy and precision. The Detection Limit (based on a signal: noise ratio of 3 : 1) achievable by this method is approximately one order of magnitude lower for each compound (i.e. approximately 50 μg kg^{-1}).

The method has been applied to the statutory testing of National Surveillance Scheme samples in NI.

In the future, this method could be applied to the study of possible carryover of both CBX and OQX into unmedicated feed. Such a study would allow National Authorities to be aware of possible contamination of unmedicated feedingstuffs with CBX or OQX, and subsequently the potential for causing accidental and/or unwarranted QCA residue levels in animals at slaughter.

6.3. Confirmation of quinoxaline carboxylic acid in tissue

Under EU legislation, Members States are required to analyse edible meat products for the presence of drug residues. In pigs, liver and gastrointestinal enzymes metabolise CBX to undetectable concentrations within a 24 to 48 hours of the cessation of medication. Therefore the metabolite, QCA, has been assigned the marker residue of CBX use in tissue, and is detectable in liver samples 6 weeks after the cessation of medication. There is a current requirement within the EU for a fast, robust, confirmatory method for QCA in porcine tissue that will meet all EU criteria for method validation.

The first objective of the work in Chapter 3 was to develop such a confirmatory method for QCA. An electrospray tandem MS method is described for QCA in porcine liver, with the use of a deuterated form of QCA (d_4-QCA) as an internal standard. This method involved the alkaline hydrolysis of the matrix followed by two liquid-liquid extractions. Interferants were separated from the analyte by use of automated cartridge based solid phase extraction. A further liquid-liquid extraction was applied to remove the analyte into an organic medium for subsequent sample concentration and preparation for HPLC. The HPLC system employing an isocratic mobile phase of methanol-water-acetic acid (40 : 59.6 : 0.4 v/v), was connected *via* electrospray interface to the MS where the analytes were detected by tandem MS in positive mode. The molecular ion $[M + H]^+$ of QCA (*m/z*

175) produces three prominent products at *m/z* 129, 102 and 75. The daughter ion at *m/z* 102 was used to calculate the QCA concentration in the sample by comparison to the internal standard (d_4-QCA) ion, *m/z* 106, within the same sample and the standards. The method was validated according to current EU legislation over the concentration range 3.0 – 300 μg kg^{-1} to reflect the spread of results in porcine liver gathered by the NI National Surveillance Scheme. The assessed detection parameters - CCα and CCβ, were 0.16 and 0.27 μg kg^{-1}, respectively. These are lower than any estimated MRPL (~6.0 μg kg^{-1}) that will be set by the EU.

The second objective of this chapter was to compare the developed method to the current in-house GC-MS method to assess its suitability and to guarantee the consistent accuracy of results. Both methods gave good agreement (r^2=0.9799) over the wide range of concentrations measured (6.5 – 213 μg kg^{-1}).

The fast and robust LC tandem MS method has been successfully applied to the statutory testing of National Surveillance Scheme samples in NI.

6.4. Bioavailability of quinoxaline carboxylic acid in pigs

Using the nitrofuran antibacterial, furazolidone, McCracken *et al.* (2000) demonstrated that even brief exposure to the excretions of previously medicated animals could result in violative residue levels in unmedicated animals. This leads to the possibility that one producer, presenting contaminated animals, could be penalised in court because of abuse by another. In order to aid National Authorities discriminate between such cases, pharmacokinetic studies were carried out to determine if the same violative residues levels

could be seen through CBX medication of pigs and the subsequent contamination of housing.

The first objective of the work described in Chapter 4 was to establish if the metabolite of CBX, QCA, could be transferred from medicated to untreated animals through exposure to a contaminated environment. A group of pigs was fed CBX infeed (50 mg kg^{-1}) for seven days and then removed from their lairage. Clean untreated pigs were moved into the same housing after cleaning. Within 2 hours of being exposed to the housing, untreated pigs showed detectable concentrations ($\leq$2 μg kg^{-1}) of QCA in liver, kidney, bile and urine samples, with significant levels after 24 hours of exposure. Thus, even a short exposure time ($\leq$ 2 hours) could result in a possible 'violative' laboratory result for CBX use.

Having shown that such transfer is possible, the second objective of chapter 4 was to determine if it was possible to differentiate in the laboratory, between animals subject to deliberate medication and those exposed to a contaminated environment. The CBX medicated animals were sacrificed at intervals over a 14-day withdrawal period. The mean urine : liver QCA concentration ratio in medicated animals subject to any form of withdrawal was found not to exceed 0.56 ± 0.21 (Mean ± SEM). The mean urine : liver QCA concentration in untreated animals, exposed to contaminated housing was found not to go beneath 10.59 ± 7.74 (Mean ± SEM). These results suggest that it is possible to differentiate between environmental contamination and deliberate medication on the basis on the urine : liver QCA concentration ratio. Tentative discriminatory criteria are suggested; CBX medicated animals that are subject to any withdrawal period would have a urine : liver QCA concentration of less than 0.80, whereas animals exposed to an environment contaminated with QCA would have a urine : liver QCA concentration

greater than 4.5. Any samples falling within the range 0.9 - 4.4 should therefore be considered as inconclusive and subject to further investigation.

These criteria are based on a small number of animals. In the future, this study could be expended to allow a more accurate definition of these tentative limits with larger groups of animals and subsequently a larger number of results from different laboratories over a longer period.

6.5. Dual confirmation of quinoxaline carboxylic acid and methyl quinoxaline carboxylic acid in tissue

In pigs, liver and gastrointestinal enzymes degrade OQX to undetectable concentrations 48 to 72 hours after the cessation of medication. Thus, the metabolite, MQCA, has been assigned as the marker residue for OQX use in liver. This allows the detection of OQX use in pigs several weeks after the cessation of medication. A current requirement within EU is for a rapid, sensitive confirmatory method for MQCA in porcine tissue that will meet all EU criteria for method validation.

The aim of Chapter 5 was the development of a method for the confirmation of OQX use. Due to the chemical and structural similarity of QCA and MQCA, the extraction method previously described in Chapter 3 was adapted to include MQCA. This allowed the dual confirmation of both QCA and MQCA, the marker residues in liver for the use of CBX and OQX, respectively, within the same sample. Deuterated forms of both compounds (d_4-QCA and d_7-MQCA) were used as internal standards to improve the robustness of the method. The samples were subject to protease enzymatic digest, followed by two liquid-liquid extractions, clean up *via* solid phase extraction, and a further liquid-liquid extraction

for concentration and preparation for HPLC. Narrow bore HPLC columns and gradient chromatography was employed to separate both compounds. Analysis was then carried out using HPLC linked *via* electrospray interface to tandem MS. The molecular ion $[M + H]^+$ of QCA (*m/z* 175) produces two prominent product ions at *m/z* 102 and 75. The daughter ion, *m/z* 102, was used to calculate the QCA concentration by comparison to the internal standard (d_4-QCA) ion, *m/z* 106, within the same sample and the standards. The molecular ion $[M + H]^+$ of MQCA (*m/z* 189) produces two prominent product ions at *m/z* 145 and 102. The daughter ion, *m/z* 145, was used to calculate the MQCA concentration by comparison to the internal standard (d_7-MQCA) ion, *m/z* 152, within the same sample and the standards.

The method was again validated according to current EU legislation. The accuracy, and precision of the method were demonstrated over the concentration range 3.0 – 150 μg kg^{-1} for both analytes, to reflect the possible range of naturally incurred samples to be measured. An MRPL for QCA or MQCA has not yet been set. Thus, the method performance characteristics were assessed at a lower than expected concentration (2.0 μg kg^{-1}) for both compounds; ensuring the method will meet any MRPL finally adopted by the EU. The assessed detection parameters, CCα and CCβ have been calculated at 0.34 and 0.57 μg kg^{-1}, respectively, for QCA and at 0.82 and 1.44 μg kg^{-1}, respectively, for MQCA.

This fast and robust method is to be applied to the statutory testing of National Surveillance Scheme samples in NI.

In the future, the developed method could be applied to establish if the metabolite of OQX, MQCA, can be transferred from medicated to untreated animals through exposure to a contaminated environment as laid out for QCA in Chapter 4. This would allow National Authorities to determine if a confirmed laboratory result for the presence of MQCA indicates conclusively the use of OQX by a producer. If such animal to animal transfer of residues is possible, the study could be extended to subsequently determine if it is possible to differentiate in the laboratory between animals subject to deliberate medication and those exposed to a contaminated environment.

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